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ACTIVITY OF PHOSPHATASES IN FRESH AND DIALYZED EXTRACTS OF NORMAL MOUSE LIVER AND OF MOUSE HEPATOMA[1]

By JESSE P. GREENSTEIN, *principal biochemist*, CHARLES E. CARTER, *assistant surgeon*, and FLORENCE M. LEUTHARDT, *junior biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

It has been shown by Greenstein, Carter, Chalkley, and Leuthardt (*1*) that (1) the rate of splitting of inorganic phosphate from ribosenucleate and from desoxyribosenucleate is much higher in digests of fresh extracts of mouse hepatomas 587 and 98/15 than of normal mouse liver; (2) more inorganic phosphate is split from ribosenucleate in dialyzed than in fresh extracts of the normal liver, spleen, pancreas, brain, and muscle of the rat, whereas no inorganic phosphate is split from desoxyribosenucleate in salt-free, dialyzed extracts of these tissues; (3) in the presence of added salts, inorganic phosphate is split from desoxyribosenucleate to a much greater extent in dialyzed than in fresh extracts of normal tissues; and (4) the rate of splitting of inorganic phosphate from adenylic, guanylic, cytidylic, and uridylic acids is the same in fresh as in dialyzed (salt-free or salt-treated) extracts of normal rat tissues. The fact that more inorganic phosphate is found in digests of the nucleates in dialyzed than in fresh extracts of the tissues has been tentatively explained on the basis either of the presence of phosphate acceptors or of nucleophosphatase inhibitors in the fresh extracts as contrasted with the dialyzed extracts (*1*). Such factors are apparently lacking in the case of the phosphatase or phosphatases for the nucleotides.

The present paper is concerned with the following questions: (1) Does the phenomenon of the increase in inorganic phosphate found in digests of the nucleates in dialyzed over that in fresh extracts of normal liver hold also for the hepatoma? (2) Does the same phenomenon hold in the case of other types of substrates which split off inorganic phosphate? The answers to these questions reveal the unique properties of the nucleates in the respects studied, as well as the marked difference between liver and hepatoma in metabolizing the nucleates.

[1] Received for publication July 19, 1946.

EXPERIMENTAL PROCEDURE

Hepatoma 587 in strain A mice and the livers of normal strain A mice comprised the tissues studied. The tissues were excised from the freshly killed animals; necrotic areas were removed from the tumors and discarded; and the materials were ground to a paste with sand in a glass mortar. The ground tissues were extracted with distilled water, centrifuged clear of debris and sand, and adjusted to a concentration with distilled water within the range where the rate of splitting of the particular substrate studied was proportional to the dilution of the extract. The final extracts were divided in halves, and one-half was dialyzed against distilled water for 24 hours at 5° C.

The digests consisted of 1 cc. tissue extract at the concentration designated plus 1 cc. substrate solution in water. The substrates in milligrams per cubic centimeter and the nucleotides in moles were as follows: disodium phenylphosphate, 1.09 (complete splitting, 155 micrograms P); sodium β-glycerophosphate, 0.97 (complete splitting, 160 micrograms P); sodium pyrophosphate, 2.2 (complete splitting, 320 micrograms P); all ribosenucleotides at 3.5×10^{-3} M (complete splitting, 109 micrograms P); ribosenucleate and desoxyribosenucleate, 5 (complete splitting, 440 micrograms P); magnesium chloride and calcium chloride were added to the digests in final concentration of 0.01M. The nucleotides were first neutralized with sodium hydroxide before addition to the extracts. The nucleates were used as the sodium salts and were described previously (*1*). The alkaline pH of 9.6 for the phenylphosphatase and β-glycerophosphatase was adjusted by adding sodium barbiturate to the stock substrate solutions. The acid pH of 4.6 was adjusted by adding an acetate buffer at 0.1M to the stock substrate solutions. The buffering at pH 6.8 was that due to the tissue extracts alone and represents the initial pH of the extracts.

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After the appropriate period of incubation, 1 cc. of 10 percent trichloracetic acid was added to each digest, the mixture was centrifuged, and the inorganic phosphate was determined in the clear supernatant solution by the method of Fiske and Subbarow (cf. *1*). The activity of the phosphatase for each substrate was estimated in terms of the micrograms of inorganic phosphate noted in the digests above that in the controls (extracts plus water). The substrates alone were stable. The data on various substrates under different experimental conditions are given in table 1.

TABLE 1.—*Phosphatases in fresh and in dialyzed extracts of normal mouse liver and of transplanted mouse hepatoma 587*

Substrate	pH	Amount of tissue per cubic centimeter of extract	Incubation time	Salt	Activity of appropriate phosphatase			
					Liver		Hepatoma	
					Fresh	Dialyzed	Fresh	Dialyzed
		Mg.	*Hours*					
Phenylphosphate	4.6	83	2	Mg	77	68	58	57
				Mg	77	70	58	62
				Ca	77	69	58	57
	9.6	83	2		60	66	18	20
				Mg	86	90	18	25
				Ca	64	70	17	20
					30	35	19	20
β-Glycerophosphate	4.6	83	2	Mg	30	35	16	20
				Ca	30	35	18	20
					56	60	14	20
	9.6	83	2	Mg	56	70	20	30
				Ca	56	66	12	20
					18	20	10	14
	6.8	83	2	Mg	30	40	14	19
				Ca	19	23	10	15
					0	0	0	0
Pyrophosphate	6.8	3	.5	Mg	178	180	73	66
				Ca	0	0	0	0
					50	52	40	42
Adenylic acid	6.8	42	1	Mg	60	64	60	60
				Ca	55	60	60	52
					60	64	40	42
Guanylic acid	6.8	42	1	Mg	64	68	68	70
				Ca	60	62	55	60
					60	64	42	46
Cytidylic acid	6.8	42	1	Mg	64	68	50	54
				Ca	60	62	48	50
					90	94	90	94
Uridylic acid	6.8	42	1	Mg	98	100	94	94
				Ca	90	92	88	92
					25	110	60	50
Ribosenucleate	6.8	166	5	Mg	40	130	94	100
				Ca	25	120	64	58
					28	0	66	0
Desoxyribosenucleate	6.8	166	5	Mg	40	140	84	65
				Ca	40	132	78	65

DISCUSSION

Relative Activity in Fresh and in Dialyzed Extracts

LIVER

With the exception of the nucleates, all the substrates studied yield, under the same conditions, very nearly the same amount of inorganic phosphate in fresh and in dialyzed extracts. Ribosenucleate yields much more phosphate in digests with dialyzed extracts, whether salt-free or salt-treated, than with fresh extracts. Desoxyribosenucleate yields no inorganic phosphate in digests with salt-free, dialyzed extracts, a phenomenon familiar from previous work (*1*); but on addition of salt, a very considerable amount of phosphate appears in the digest with dialyzed extract which is much greater than that which appears in the fresh extract. It would appear that of the phosphate-yielding substrates studied in extracts of normal tissues, the nucleates are unique in that they apparently yield more inorganic phosphate in dialyzed (salt-treated) extracts than in fresh extracts. The inorganic phosphate may be less in digests of the nucleates with fresh than with dialyzed tissue extracts because of the presence of dialyzable phosphate acceptors or because of the presence of dialyzable inhibitors in the fresh extracts. At the present time, it is impossible to make a choice between these alternatives. If the difference between fresh and dialyzed tissue extracts is to be ascribed to the presence of phosphate acceptors in the former, then a new role for the nucleic acids may be envisaged, namely, that of phosphate donors. In this way the nucleic acids would share with adenosinetriphosphate the property of yielding, through the action of the appropriate phosphatases, transferable phosphate to products of intermediate metabolism. Experiments to test the possibility are at present under way. If, on the other hand, the difference between fresh and dialyzed tissue extracts is to be ascribed to the presence of phosphatase inhibitors in the former, a good case could be made for the bicarbonate ion as the inhibitor implicated (*1*, *2*).

HEPATOMA

With one exception, that of desoxyribosenucleate in salt-free, dialyzed extracts, all the substrates studied yield very nearly the same amount of phosphate in dialyzed as in fresh extracts. As in the case of dialyzates of all normal tissues, the enzymatic dephosphorylation of desoxyribosenucleate in dialyzed hepatoma extracts requires the presence of activating salts. In contrast with what happens in the normal tissue, the dephosphorylation of desoxyribosenucleate in salt-treated, dialyzed, hepatoma extracts or the dephosphorylation of ribosenucleate in salt-free or salt-treated dialyzed extracts does not exceed and is nearly the same as that in fresh extracts. On the basis of the possible explanation advanced to account for the difference between fresh and dialyzed normal tissues, it would appear that the fresh hepatoma extracts are lacking in either phosphate acceptors or phosphatase inhibitors. Since the amount of inorganic phosphate in fresh extracts and under similar conditions is much higher in the hepatoma than in the liver digests of the

nucleates (table 1), it would seem that the fresh hepatoma extracts, as compared with fresh liver extracts, are already in a quasi-dialyzed or "empty" state. This is consistent with the observation of Chalkley and Greenstein on the relative capacity of extracts of liver and of hepatoma to decolorize methylene blue (*3*). Thus, the rate of decolorization of the dye is very much greater in fresh liver than in fresh hepatoma extracts; but on dialysis of the extracts, the rate of decolorization of the liver drops considerably and is practically the same as that of the hepatoma extract. Normal tissues contain a considerable reserve of various components, many of them dialyzable, which is lacking in tumor tissues. The lack of various components in tumors or perhaps the possession of them in minimal amounts by tumors is a consistent finding of numerous investigators in the biochemistry of the cancer problem. The interpretation of the present findings on the difference between the apparent dephosphorylation of the nucleates in liver and in hepatoma extracts is, therefore, not out of line with the foregoing concept.

Relative Phosphatase Activity of Liver and Hepatoma

Under similar conditions, the activity of acid phenylphosphatase and of the nucleotidases is very nearly the same in extracts of liver and of hepatoma. On the other hand, the activity of alkaline phenylphosphatase, of acid, alkaline, and neutral β-glycerophosphatase and of pyrophosphatase is greater in liver than in hepatoma extracts. The apparent dephosphorylation of the nucleates is greater in fresh extracts of the hepatoma than in fresh extracts of the liver, but the reverse is true of salt-treated, dialyzed extracts of these tissues.

At neutral pH, it would appear that the highest rate of dephosphorylation is achieved in the case of pyrophosphate, followed by the nucleotides, β-glycerophosphate, and the nucleates in that order. The phenomenally high activity of pyrophosphatase in both liver and hepatoma is remarkable. The lesser rate of apparent dephosphorylation of the nucleates, as compared with the other substrates, may be due to the preliminary degradation of the nucleates to smaller, more susceptible fragments through the action of nucleases (*2*).

Effect of Magnesium and Calcium Salts

Of the phosphatases studied (table 1), only those concerned with the splitting of the nucleates and of pyrophosphate appear to be greatly affected by the presence of electrolyte. Pyrophosphatase requires magnesium for its activation and cannot use calcium. This activating effect holds for both liver and hepatoma. The position of magnesium in respect to the activation of pyrophosphatase was pointed out by Bailey and Webb (*4*). In the case of the nucleates, calcium and magnesium appear to be nearly equally effective in producing more inorganic phosphate split from the substrate. Indeed, the acceleration of the enzymatic splitting of the nucleates is shared by a wide variety of salts, both inorganic and organic (*1, 2*).

Since the dephosphorylation of the nucleotides is not affected by the salts used, the action of these salts on the nucleates must occur at some stage or stages prior to the degradation of the nucleates to nucleotides. Magnesium and sodium salts accelerate the liberation of titratable acid groups in the two kinds of nucleate digested with the appropriate purified nuclease (*2*).

SUMMARY

The relative susceptibility to the enzymatic splitting of inorganic phosphate from different kinds of substrates was studied under various conditions in fresh and in dialyzed extracts of normal mouse liver and of transplanted mouse hepatoma 587.

The activity of the phosphatases for phenylphosphate, β-glycerophosphate, pyrophosphate, and for ribose nucleotides was nearly the same in fresh and in dialyzed extracts of either liver or hepatoma. In salt-treated, dialyzed extracts of liver the phosphate split from ribosenucleate and from desoxyribosenucleate was much higher than in fresh extracts of this tissue, whereas in salt-treated, dialyzed extracts of the hepatoma the amount of phosphate split was the same as in fresh extracts. Tentative explanations of these phenomena are discussed.

Pyrophosphatase activity in liver and in hepatoma is very high in presence of magnesium but not of calcium. The dephosphorylation of the nucleates is accelerated by calcium and magnesium. The phosphatases for the other substrates are little affected by the presence of these ions.

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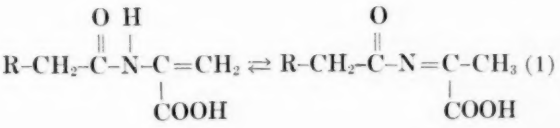
SPECTROPHOTOMETRIC DETERMINATION OF DEHYDROPEPTIDASE ACTIVITY IN NORMAL AND NEOPLASTIC TISSUES[1]

By CHARLES E. CARTER, *assistant surgeon*, and JESSE P. GREENSTEIN, *principal biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

A possible mechanism of degradation of amino acids while in peptide linkage was considered by Bergmann et al. (*1*) to involve an α, β dehydrogenation resulting in dehydropeptides. Enzymatic hydrolysis of synthetic dehydropeptides was first demonstrated in extracts of hog kidney by Bergmann and Schleich (*2*) and was recently described in a variety of normal and neoplastic tissues by Greenstein and Leuthardt (*3*), thus suggesting that such classes of compounds might indeed be biologic intermediates. Susceptible substrates for tissue dehydropeptidase have been found to possess an exo-configuration. That is, the unsaturated residue requires a free terminal carboxyl group. Possibilities for mesomerism in such a compound suggest that dehydropeptides exist as equilibrium mixtures of resonating and tautomeric structures, two of which are illustrated here:



Masking of the terminal carboxyl group by combination with another amino acid results in a compound which is not a substrate for dehydropeptidase (*2*).

Enzymatic hydrolysis of the dehydropeptides may be followed by the chemical determination of the principal products of the reaction, ammonia and pyruvic acid, or by a method reported herein based upon the disappearance of the characteristic ultraviolet-absorption spectra of dehydropeptides during enzymatic hydrolysis. In this paper the spectrophotometric method for determining dehydropeptidase activity is described, and the results of application of this method to a study of normal and neoplastic tissue extracts are reported.

MATERIALS AND METHODS

The synthetic dehydropeptide, glycyldehydroalanine, was prepared according to the directions of Bergmann, Schmitt, and Miekeley (*1*). The synthesis of this compound is accomplished with some difficulty, apparently because of frequent failures to obtain satisfactory coupling of chloracetamide with pyruvic acid in the initial step. The product of this reaction, chloracetyldehydroalanine, has an ultraviolet-absorption spectra practically identical with glycyldehydroalanine, and the purity of the product may thus be checked by determining its absorption characteristics.

The Beckman spectrophotometer was used in all determinations of ultraviolet-absorption spectra. Several spectrophotometric determinations of dehydropeptidase activity were checked with ammonia and pyruvate analyses on replicate digests according to previously described methods (*3*).

Tissue extracts were prepared by grinding organs and tumors from freshly killed animals with sand and extracting with 12 volumes of water followed by light centrifugation.

The substrate glycyldehydroalanine showed no spontaneous hydrolysis at 37°.

EXPERIMENTAL PROCEDURE

ULTRAVIOLET-ABSORPTION SPECTRA OF GLYCYLDEHYDROALANINE AND THE SPECTROPHOTOMETRIC DETERMINATION OF DEHYDROPEPTIDASE ACTIVITY

The high characteristic absorption of glycyldehydroalanine in the ultraviolet region is shown in figure 1, a finding which may be attributed to the resonance and tautomerism of the unsaturated peptide. By contrast the saturated peptide, glycylalanine, has but slight absorption. Enzymatic hydrolysis of the dehydropeptide yields only one product with a detectable absorption in the ultraviolet, namely, pyruvic acid. As illustrated in figure 1, pyruvate at concentrations equimolar with glycyldehydroalanine has a comparatively low ultraviolet absorption, especially at 2700 Å. This accounts for the observation that upon enzymatic hydrolysis of glycyldehydroalanine there is a progressive disappearance of the absorption characteristics originally exhibited by the dehydropeptide and is therefore the basis for the spectrophotometric determination of dehydropeptidase activity. Kinetic studies have revealed no intermediates of the reaction with absorption in the

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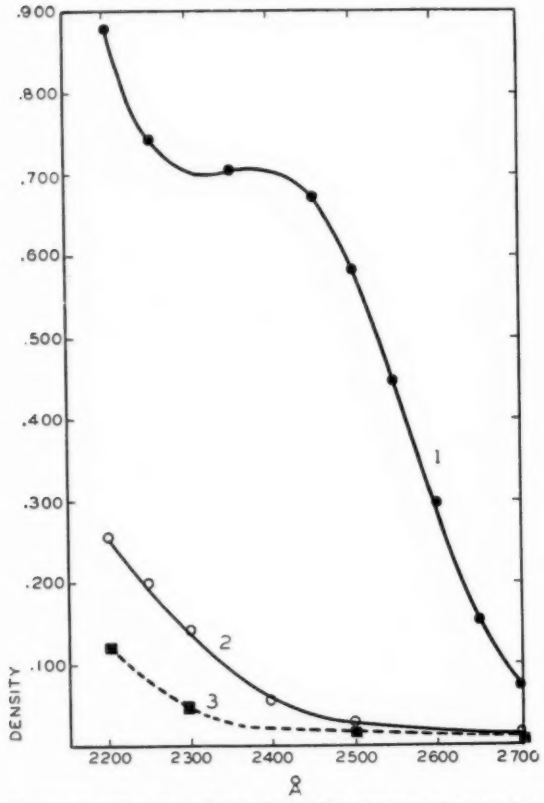


FIGURE 1.—The ultraviolet-absorption spectra of equimolar (1.7×10^{-4} M) solutions of (1) glycyldehydroalanine, (2) pyruvic acid, and (3) glycylalanine. Data derived from measurements employing 1-cm. cell.

ultraviolet region; that is, the absorption curve characteristic of the dehydropeptide progressively decreases without appearance of other maxima to assume finally absorption characteristics which may be attributed to the residual pyruvate formed.

Figure 2 illustrates several considerations fundamental to the spectrophotometric determination of dehydropeptidase activity. The conditions from which the data of this figure were derived were the following: A digest consisting of 1 cc. of rat-liver extract (6 volumes water) and 1 cc. of a solution containing 3.6 mg. of glycyldehydroalanine was set up in duplicate for chemical determination of the products of the enzymatic hydrolysis of glycyldehydroalanine, ammonia and pyruvate, and for spectrophotometric determination of dehydropeptidase activity on aliquots of the digests. Incubation period at 37° C. was 2 hours. Ammonia determinations in digests at the end of incubation period indicated 77 percent splitting of the substrate. Pyruvate, determined as 1,4-dinitrophenylhydrazone, indicated a similar extent of enzymatic hydrolysis. The 1,4-dinitrophenylhydrazone of pyruvic acid was isolated and found to have a melting point of 216°–218° (corrected) and a Kjeldahl nitrogen content of 10.5 percent in close agreement with theory. For the spectrophotometric determination of dehydropeptidase activity, the 2500Å wavelength was chosen for the following reasons: (1) The extract absorption curve here exhibits a minimum in the region where glycyldehydroalanine has a high ultraviolet absorption. Thus, at this wavelength of the total absorption of extract plus substrate only about 50 percent of the absorption under these conditions is contributed by the extract alone. This permits a considerable accuracy of determination. (2) At 2500Å the absorption of pyruvate formed in the reaction is negligible and

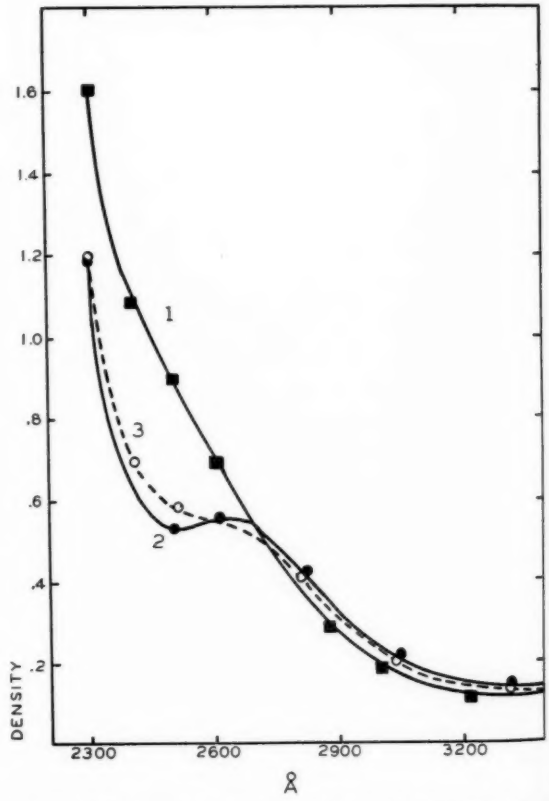


FIGURE 2.—Curve 1, Liver extract plus glycyldehydroalanine before incubation; curve 2, Absorption curve of liver extract alone; curve 3, Liver extract plus glycyldehydroalanine following 2 hours' incubation at 37° C. All samples were diluted 1:100 before reading.

may be ignored in calculations of dehydropeptidase activity. (For kinetic studies the absorption of residual pyruvate must be taken into account.) (3) Although the maximum of ultraviolet absorption of glycyldehydroalanine occurs at 2400Å and progressively disappears during incubation as does the absorption at 2500Å, in this region tissue extracts have a high absorption. In adapting the spectrophotometric method for routine use, it was found that determinations at 2400 Å read against an extract blank required prohibitively high slit widths, whereas reading at 2500 Å obviated this technical difficulty.

Under these conditions, figure 2 illustrates the spectrophotometric determination of dehydropeptidase activity. The initial difference between density of absorption of extract and extract plus substrate at 2500Å was 0.490. During incubation this difference decreased to 0.070, thus indicating approximately 80 percent hydrolysis of the substrate, a finding in agreement with chemical determination of enzymatic activity as revealed by analyses for ammonia and pyruvate.

Throughout this paper dehydropeptidase activity refers to dehydropeptidase I, a distinction based upon the observation that the distribution in tissues of the capacity to split glycyldehydroalanine (dehydropeptidase I) differs from the capacity to split chloracetyldehydroalanine (dehydropeptidase II) (*3*). The latter appears to be absent from all tumors studied. The spectrophotometric method of determination of dehydropeptidase activity may also be applied to the enzymatic hydrolysis of chloracetyldehydroalanine under suitable conditions.

For routine determination of dehydropeptidase activity, a rapid, accurate, and easily performed method has been adopted. Tissues are extracted with 12 volumes of water after grinding with sand, and debris is removed with light centrifugation. Two-tenths of a cubic centimer of extract is added to 0.2 cc. of a solution containing 0.72 mg. of glycyldehydroalanine, and the digest is incubated for various periods. Blanks containing water in place of glycyldehydroalanine and glycyldehydroalanine alone are run as controls. (The substrate in aqueous solution has been found to be stable for days at room temperature.) Following incubation, 10 cc. of distilled water is added to digest and to extract blank, and the digest is read against the extract blank at 2500 Å. Under these conditions, initial density readings of 1.4 are obtained, and during incubation there is a progressive decrease of this value to almost zero (0.005–0.010).

Figure 3 illustrates the application of this method to various tissues for increasing periods of incubation. These data illustrate the finding that kidney possesses the highest activity, and human serums low but nevertheless definite activity.

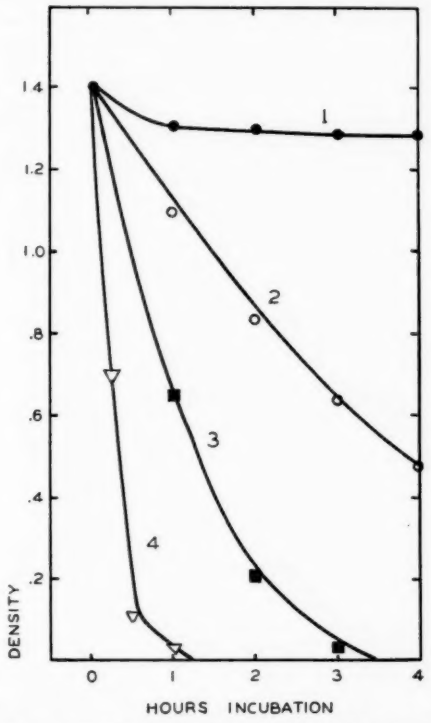


FIGURE 3.—Experimental conditions described in text; 1, human serums; 2, rabbit serums; 3, rat liver; 4, rat kidney.

Calculations of dehydropeptidase activity may be made from the linear part of the hydrolysis curve. A convenient statement of activity which we have used is based on the amount of total nitrogen in 0.2 cc. of extract and is expressed as milligrams of glycyldehydroalanine split per hour per milligram tissue-extract nitrogen. (See table 1.)

DEHYDROPEPTIDASE I ACTIVITY OF NORMAL AND NEOPLASTIC TISSUES

Table 1 is a summary of the dehydropeptidase I activity of several normal and neoplastic tissues determined according to the spectrophotometric method given previously. The uniformly high dehydropeptidase I activity of all tumors examined is a striking but not unique finding among enzymes concerned in the protein metabolism of tumors. Maver and Dunn (*4*) reported high catheptic activities for a variety of tumors studied. However, the rapid enzymatic hydrolysis of the dehydropeptides in normal and neoplastic tissues

stands in marked contrast with the comparatively inactive systems which split saturated peptides (*5*).

TABLE 1.—*Dehydropeptidase I activity of normal and neoplastic tissues*

Tissue [1]	Total N in extract	Glycyl-dehydro-alanine hydrolyzed in 1 hour	Glycyldehydro-alanine split per hour per milligram extract N
Mouse:	*Milligrams*	*Milligrams*	*Milligrams* $\times 10^{-2}$
Liver	0.55	0.50	90
Kidney	.50	[2] 1.34	268
Spleen	.43	.49	114
Brain	.32	.36	110
Muscle	.25	.20	80
Spontaneous mammary tumor	.32	.64	200
CR 180	.31	.29	93
S 37	.30	.40	135
Spindle-cell sarcoma	.27	.39	144
Transplanted mammary tumor	.33	.62	188
Do	.27	.38	141
Transplanted hepatoma 587	.36	.63	175
Rat:			
Liver	.40	.38	92
Fetal liver	.31	.34	110
Regenerating liver	.40	.30	74
Primary hepatoma	.20	.37	170

[1] All extracts were made with 12 volumes of distilled water.
[2] Based on ½-hour incubation.

STUDIES ON PURIFIED DEHYDROPEPTIDASE I FROM RABBIT KIDNEY

A simple purification of dehydropeptidase I, resulting in a sixfold increase in activity was effected by separating the pseudoglobulin fraction of rabbit kidney through precipitation of an aqueous extract (10 volumes water) with half-saturated ammonium sulfate, followed by dialysis against tap and distilled water, and centrifugation to remove euglobulin and debris. This fraction exhibited maximal activity in the absence of salt and was inhibited to varying degrees by addition of neutral phosphate buffer (pH 7.1), an effect proportional to concentration of buffer (fig. 4). A similar effect was observed upon addition of sodium chloride and magnesium sulfate (fig. 5).

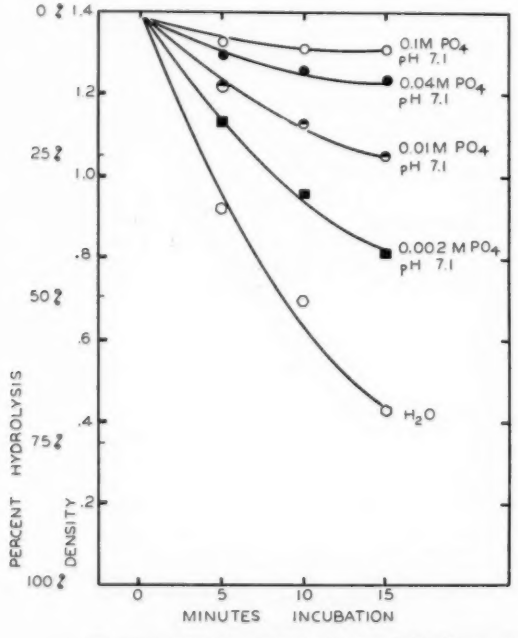
The peptidase which splits benzoylarginine-amide, like dehydropeptidase I, is considerably inhibited by neutral phosphate (*6*).

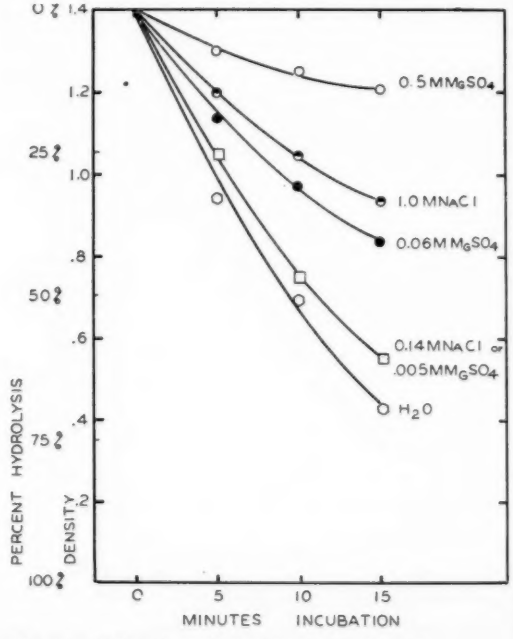
The finding of salt inhibition of dehydropeptidase I activity complicates the determination of pH optima of enzyme activity in adequately buffered solutions and is another example of the inadvisability of indiscriminate use of phosphate buffers in determination of enzyme activity.

Fractionation of rabbit serum revealed dehydropeptidase I activity solely in the pseudoglobulin moiety.

DISCUSSION

The finding of high dehydropeptidase I activity in normal and neoplastic tissues argues for the role of dehydropeptides as biologic intermediates in protein metabolism. The nature of the

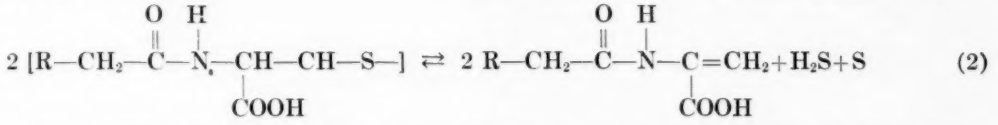




FIGURES 4 (left) and 5 (right).—Inhibitory effect of salts on activity of purified dehydropeptidase I.

precursors for such a class of compounds remains a matter of conjecture. It has been demonstrated that dehydropeptides which are susceptible to enzymatic hydrolysis possess an exo-configuration. This limits consideration of natural precursors to several groups.

(1) That peptides of cystine or cysteine could give rise to dehydropeptides was suggested by Greenstein and Leuthardt (3). Such a reaction involves the splitting of sulfur by the enzyme desulfurase which results in an unsaturated residue according to equation (2).



(2)

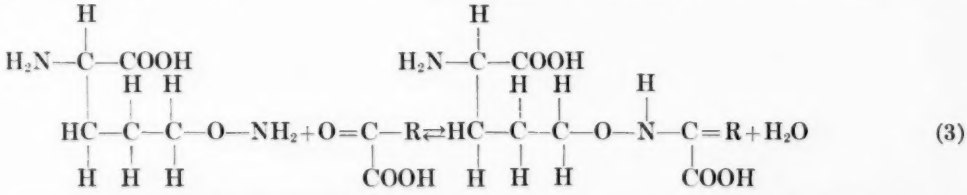
The unsaturated peptide is then split by dehydropeptidase to ammonia, pyruvate, and an acyl amino acid residue, products identical (with the exception of sulfur and hydrogen sulfide) with those formed upon enzymatic hydrolysis of synthetic dehydropeptides.

Restricting the role of cystine peptides as natural precursors is the limited distribution of the enzyme which accomplishes the desulfuration of the peptide as opposed to the widespread activity of dehydropeptidase, i. e., tumors which possess no desulfurase activity have uniformly high dehydropeptidase activity.

(2) A similar argument may be advanced for peptides of serine (threonine?). However, glycyl-*d*, *l*-serine was found to be relatively unsusceptible to enzymatic degradation [2] in tissue extracts.

(3) It is possible that dehydropeptides arise from the rupture of carbon-carbon bonds during the intermediary metabolism of proteins. Such an in vitro reaction is without precedent and must be considered unlikely.

(4) Condensation of an amino acid amide (i. e., glutamine) free or in peptide linkage, with an α-keto acid to form the corresponding dehydropeptide according to the following scheme as suggested by Bergmann and Fruton (7).



(3)

Implicit in such a scheme is the splitting by dehydropeptidase of the peptide linkage involving the γ carbon atom of glutamic acid. This type of linkage is not susceptible to peptidases acting upon known saturated analogs. However, in support of such a scheme (3) is the fact that glutathione involves a peptide bond at the γ carbon atom of glutamic acid.

If dehydropeptides occur as biologic intermediates in protein metabolism (as yet the only evidence for this is the finding of widespread enzymatic activity which accomplishes the hydrolyses of dehydropeptides of exo-configuration), then one must elucidate the origin of this class of compounds. Proof of the existence of dehydropeptides as intermediates of protein metabolism would be of primary importance. Preliminary attempts to demonstrate the characteristic absorption spectra of the dehydropeptide arising during enzymatic hydrolysis of cystine peptides have so far been unsuccessful, probably due to the transient nature of the unsaturated intermediate as compared with the relatively slowly metabolized parent substance.

SUMMARY

The ultraviolet-absorption spectrum of the dehydropeptide, glycyldehydroalanine, and the spectrophotometric determination of dehydropeptidase I activity based on the disappearance of the high characteristic absorption of the compound at 2500 Å during enzymatic hydrolysis are described.

Purification of dehydropeptidase I activity of rabbit kidney was achieved by isolation of the pseudoglobulin fraction. Neutral solutions of salts were found to inhibit dehydropeptidase I activity, an effect proportional to concentration of salt.

Primary and transplanted mouse and rat tumors were found to possess uniformly high dehydropeptidase I activity.

Some theoretical aspects concerning the biologic origin of dehydropeptides are discussed.

[2] Unpublished observations.

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INFLUENCE OF α KETO ACIDS ON THE DESAMIDATION OF AMINO ACID AMIDES[1]

By JESSE P. GREENSTEIN, *principal biochemist*, and CHARLES E. CARTER, *assistant surgeon, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

Among the possible mechanisms whereby dehydropeptides may be formed in tissues is that involving the condensation of an amino acid amide with an α keto acid (*1*):

$$RCONH_2 + R'COCOOH \rightarrow RCON{=}C(COOH)R' \quad (1)$$

Such a condensation is used for the mild in-vitro synthesis of this type of compound, and since it involves chemical groups of known biologic occurrence, it does not seem improbable of being at least one of the mechanisms whereby the dehydropeptides are formed in tissues. The dehydropeptides in turn are rapidly split in tissues by dehydropeptidases to products which include ammonia and the corresponding α keto acid (*2-5*):

$$RCON{=}C(COOH)R' \rightarrow RCOOH + NH_2 + R'COCOOH \quad (2)$$

The net results of the successive reactions 1 and 2 are therefore the desamidation of the amino acid amide and the recovery of the original keto acid. The latter thus plays the role of a cosubstrate.

Among the ways in which this hypothesis can be tested is to incubate mixtures of tissue preparations (1) with amino acid amides alone, and (2) with amino acid amides and added α keto acids. If the scheme as presented is correct, the rate of ammonia produced from (2) should be greater than from (1), and the keto acid recovered at the end of the incubation period should be the same in the presence as in the absence of the amides. In this connection, the possibility of transamination reactions involving the transfer of ammonia from the amino acid amide to the α keto acid must also be considered, and the oxidative desamination of the amino acid so formed must be known.

We have employed in this study glutamine and asparagine as the amino acid amides, together with pyruvic acid as the α keto acid. These substrates were chosen because they are widespread in most animal and plant tissues. Implicit in the reactions 1 and 2 involving these compounds are (1) the condensation by some enzymatic process of the keto acid with the acid amide groups at the γ carbon of glutamine and at the β carbon of asparagine, and (2) the subsequent splitting of the dehydropeptides by action of dehydropeptidase. These types of peptide linkages are not susceptible to peptidases which act upon known saturated peptide analogs. In support of the biologic occurrence of the γ peptide linkage, however, is the presence of glutathione in tissues.

We have further extended the testing of the condensation hypothesis of dehydropeptide formation (equation 1) by employing the peptide, pyruvoylglycine, in place of pyruvic acid. If our scheme is correct, the condensation product would be the tripeptide, glutamyl- or aspartyldehydroalanylglycine, in which the dehydroalanyl residue is in the middle of the chain. According to Bergmann and Schleich (*2*), such dehydropeptides, in which the dehydro residue is in the middle of the chain, are not attacked by dehydropeptidases. Therefore, the addition of pyruvoylglycine to the amino acid amides in the presence of tissue extracts, even though the dehydropeptide condensation occurred, should lead to little or no increase in ammonia production. This is, of course, also dependent upon the rate of condensation of the amide with pyruvoylglycine being much greater than the rate of hydrolysis of the pyruvoylglycine. Bergmann and Grafe (*6*) have showed that the enzymatic splitting of pyruvoylglycine by pancreatin is extremely slow.

The experiments whereby these concepts have been tested are described in the present paper.

EXPERIMENTAL PROCEDURE

Tissue extracts were prepared exactly as before (*3, 4*). Each cubic centimeter contained the equivalent of 333 mg. of tissue. The amino acids and amino acid amides were made up to 1.4×10^{-2} M stock solutions. The keto acids were made up to 2.7×10^{-2} M stock solutions, unless otherwise noted. All stock solutions were made up fresh before each run and were brought to pH 7 with dilute sodium hydroxide.

Considerable difficulty was experienced in the final step of the synthesis of pyruvoylglycine, namely, that involved in the preparation of α-α diacetaminopropionic acid by the condensation of acetamide with pyruvic acid (*1*). This step was accomplished by recourse to the method of Böttinger (*7*) and consists in the combination of acetonitrile with pyruvic acid in concentrated sulfuric acid. Since this method is not fully described by Böttinger for this system, a brief description of the procedure employed is given.

Four hundred cubic centimeters of concentrated sulfuric acid was chilled, with stirring, to 10° C., and then 77 gm. of pyruvic acid was added. With good stirring, 100 gm. of acetonitrile was slowly added, care being taken that the temperature did not rise above 50°. The reaction took about 1 hour. The mixture was cooled to 10° and then

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poured into 4 l. of shaved ice. Intense chilling took place, and the reaction product crystallized out. When the ice had melted, the precipitate was filtered off and washed thoroughly with cold water. A single crystallization from alcohol yielded pure diacetaminopropionic acid. The remainder of the synthetic steps toward the preparation of pyruvoylglycine were straightforward, and the final product after recrystallization from ether-petroleum possessed all the properties of pyruvoylglycine, as described by Bergmann and Grafe (*6*), that is, a melting point of 90° nitrogen content of 10.0 percent (theoretical, 9.7 percent), and the faculty of turning a mixture of ammoniacal nitroprusside to a blue color followed by a muddy green color on addition of potassium hydroxide.

The digests were prepared by mixing 1 cc. of the extract with 1 cc. of amino acid or amino acid amide plus either 1 cc. of keto acid or 1 cc. of water. Control digests contained only the tissue extracts plus added water to give the usual volume. The substrate solutions were entirely stable. The ammonia evolved in the solutions was determined by the method described (*3*), and the enzymatic activity was established by noting the excess of ammonia found in the test solutions over that found in the control solutions.

The first experiments were designed to observe the rate of desamidation of glutamine in the presence of pyruvate (table 1). The desamidation rate in extracts of the hepatoma was less than in those of normal liver. In 2 hours under these conditions, glycyldehydroalanine is nearly completely hydrolyzed (*3*, *4*).

TABLE 1.—*Time course of evolution of ammonia in digests of rat liver and hepatoma extracts with glutamine and pyruvate* [1]

Digestion period (in hours)	Ammonia N evolved [2]	
	Liver	Primary hepatoma
	Micrograms	*Micrograms*
1	30	4
2	50	10
3	80	16
4	100	24
5	110	31

[1] Mixtures composed of 1 cc. amino acid amide plus either 1 cc. water and 1 cc. keto acid, plus 1 cc. extract equivalent to 333 mg. tissue. Glutamine, 1.4 x 10^{-2}M stock solution. Pyruvate, 2.7 x 10^{-2}M stock solution.
[2] In terms of micrograms. Theoretical maximum amide nitrogen, 196 micrograms, determined by acid (1NHCl) hydrolysis.

The data in table 2 show (1) that the relatively weak desamidation of glutamine and asparagine is considerably increased when pyruvate is added to the mixture, but that pyruvoylglycinate has little or no effect in this regard, (2) that pyruvate has very little effect on the desamidation of glutamate or aspartate, (3) that alanine and alanylglycine yield relatively little ammonia under these conditions, and (4) that the hepatoma is much weaker than liver in reacting to added pyruvate.

TABLE 2.—*Ammonia nitrogen evolved in digest of rat liver and hepatoma extracts with various substrates*

Substrates [1]	Ammonia N evolved [2]	
	Liver [3]	Primary hepatoma
	Micrograms	*Micrograms*
Glutamine	9	10
Glutamine+pyruvate	102	22
Glutamine+pyruvoylglycinate	12	10
Glutamate	3	2
Glutamate+pyruvate	4	4
Glutamate+pyruvoylglycinate	4	3
Asparagine	22	12
Asparagine+pyruvate	135	14
Asparagine+pyruvoylglycinate	36	13
Aspartate	8	2
Aspartate+pyruvate	12	4
Aspartate+pyruvoylglycinate	9	3
Alanine	10	0
Alanylglycine	0	0
Pyruvate	1	0
Pyruvoylglycinate	0	0

[1] [2] Same as in table 1. Incubation period 4 hours at 37° C.
[3] Dialyzed liver extract incubated under the same conditions with glutamine plus pyruvate yielded 90 micrograms of ammonia nitrogen as compared with a value of zero for the same mixture minus pyruvate.

The ammonia noted in rat-liver extracts in the presence of pyruvate is invariably higher than in the absence of pyruvate. Determinations of the amount of pyruvate in the digests of rat-liver extract with glutamine, asparagine, etc., at the end of the incubation period indicated relatively little decrease in the concentration of the pyruvate, as shown in the following tabulation: [2]

Mixture	*Pyruvate* [1] *(in milligrams)*
1 cc. extract + 1 cc. pyruvate + 1 cc. water	2.2
1 cc. extract + 1 cc. pyruvate + 1 cc. glutamine	2.2
1 cc. extract + 1 cc. pyruvate + 1 cc. asparagine	2.2

[1] Initial concentration of pyruvate was 3.0 mg.

Studies of the pyruvate effect on the ammonia production from glutamine were instituted with extracts of various rat tissues, as given in table 3. It is clear that kidney has a powerful glutaminase, and that pyruvate increases still further the yield of ammonia, but the percentage increase in ammonia production on the addition of pyruvate to the mixture of tissue extract and glutamine is greatest in the case of liver.

The data in the following tabulation show that an optimal effect of pyruvate on the desamidation of glutamine is achieved in digests with rat liver at a concentration of about 2.5 mg. of pyruvate per cubic centimeter of stock solution, or roughly

[2] The extract concentration was equivalent to 333 mg. tissue per cubic centimeter; the pyruvate concentration, 3 mg. per cubic centimeter stock solution; and amino acid amides at 1.4 x 10^{-2} M stock solution. Pyruvate was determined as 2:4 dinitrophenylhydrazone as before (*3*).

TABLE 3.—*Ammonia nitrogen evolved in digests of rat tissue extracts with various substrates* [1]

Tissue	Ammonia N evolved from [2]—	
	Glutamine	Glutamine and pyruvate
	Micrograms	*Micrograms*
Liver	10	98
Kidney	120	183
Spleen	27	32
Brain	20	40
Pancreas	45	47
Muscle	9	25

[1] [2] Same as in table 1. Incubation period 4 hours at 37° C.

2.7×10^{-2} M. The ammonia nitrogen was evolved under the same conditions as in table 1, except for the changing concentrations of pyruvate.

Milligrams pyruvate per cubic centimeter stock solution:	*Ammonia N evolved (in micrograms)*
10	18
5	88
2.5	100
1.25	90
0.62	72
0.31	48
0.15	23
0.07	18
0.03	10
0.015	10
0.008	10
0.004	10
0.002	10
0.001	10
0.0	10

In order to see whether the ammonia evolved in mixtures of glutamine with pyruvate is accompanied by a corresponding decrease in the amide nitrogen of the glutamine, experiments were performed in which free ammonia and amide nitrogen were determined on aliquots of the same digest. The amide nitrogen was determined by treating the digest with 1 cc. of 5 percent trichloracetic acid, centrifuging off the precipitated protein, and adding concentrated hydrochloric acid to 1N concentration. The mixture was then gently boiled for 5 minutes, cooled, treated with alkali, and aerated in the usual manner into sulfuric acid traps, which were subsequently nesslerized. The values of ammonia so obtained were the sum of the free ammonia formed by enzymatic desamidation of the amide plus the residual amide nitrogen of the glutamine. When by independent measurement on another aliquot of the same digest, the amount of free ammonia formed enzymatically is known, the residual amide nitrogen can be calculated by difference. The results noted in table 4 show that the increase in free ammonia in the presence of pyruvate is accompanied by a corresponding decrease in amide nitrogen of the glutamine.

TABLE 4.—*Change in amide nitrogen of glutamine incubated with pyruvate in presence of rat liver extracts* [1]

Substrates	Period of incubation	Ammonia N	Residual amide N	Total N [2]
	Hours	*Micrograms*	*Micrograms*	*Micrograms*
Glutamine	0		190	190
Glutamine + pyruvate	0		190	190
Glutamine	4	6	182	188
Glutamine + pyruvate	4	98	88	186

[1] Mixtures prepared as in table 1.
[2] Theoretical maximum amide nitrogen 196 micrograms.

DISCUSSION

The data in table 2 support but do not necessarily prove the concepts of the mechanisms postulated in the Introduction, that the α keto acids exert their effect upon the desamidation of the amino acid amides (1) by condensing to form a dehydropeptide, and (2) by the subsequent splitting of the susceptible dehydropeptide by dehydropeptidase. The net result is the desamidation of the amino acid amide and the recovery of the original keto acid in nearly the same concentration in the presence or absence of amide. It is probable that the dehydropeptide is split as fast as it is formed, and hence changes in pyruvate concentration would not be readily observable. The final proof of the hypothesis will involve among other things the reproduction of the findings with purified enzyme systems.

Neither alanine nor alanylglycine yields much ammonia under the conditions used, which eliminates the possibility that transamination reactions may be concerned to any significant extent. Virtanen and Laine (*8*) noted that the transamination of pyruvic acid with glutamine and asparagine occurred after the amide group was split, and thus the amide group does not participate in the transamination reactions (enzymes from vegetable sources).

The enormous rise in ammonia on adding pyruvate to a digest of glutamine or asparagine in rat-liver extract is rather extraordinary and to our knowledge has not been reported before. That this ammonia is derived almost completely from the amide group is suggested by the relatively small amount of desamination which takes place with glutamate or with aspartate, whether pyruvate is present or not (table 2), and by the corresponding decrease in amide nitrogen of glutamine (table 4).

There is a definite asparaginase in rat liver which appears to be even more active than the accompanying glutaminase. This finding has been consistently observed.

The figures given in table 2 for the ammonia noted in digests of either glutamine or asparagine with pyruvate, 102 or 135 micrograms of ammonia

nitrogen, respectively, represent roughly one-half of the theoretical amide nitrogen available. The data in table 1 suggest that further incubation beyond this 4-hour period would result in the production of more ammonia, but the reaction was not carried beyond 5 hours.

The relatively small effect of pyruvate on the desamidation of glutamine and of asparagine in extracts of the primary hepatoma, as compared with the magnitude of this effect in extracts of normal liver, is interesting in view of the fact that the dehydropeptidase activity of the tumor is greater than that of the liver (*5*). If the mechanisms of ammonia production are actually as postulated in the Introduction, then it would appear that it is the primary condensation of the amides with pyruvate which is relatively weak in the tumor.

The failure of pyruvoylglycine to lead to increased ammonia production may be explained on the basis either of failure to condense with the amide or of actual condensation with the amide to form a tripeptide, in which the dehydroalanyl residue is in the middle of the chain and hence unreactive toward dehydropeptidase (*2*). Such small increases in ammonia production as noted in table 2 might have been due to a slight hydrolysis of pyruvoylglycine to pyruvic acid and glycine.

Implicit in the concepts advanced is the formation of a peptide bond at the γ carbon of glutamic acid and at the β carbon of aspartic acid. The former type of bond is present in glutathione. Condensation of glutamine in the fashion indicated with pyruvoylglycine would lead to a desthiolglutathione, and work on this possibility is in progress.

The increase in ammonia in the digests of glutamine with pyruvate in extracts of tissues stands in marked contrast with the disappearance of added ammonium salts in the presence of pyruvate and tissue slices. Thus, slices of bird liver form glutamine from ammonium salts and pyruvate (*9*), and urea is formed from ketoglutaric acid and ammonium salts in the presence of rat-liver slices (*10*). In connection with the last-mentioned experiments, it was noted that urea appears to be synthesized from glutamine and ammonium salts plus a source of carbon dioxide.

SUMMARY

Aqueous extracts of certain normal rat tissues exhibit a relatively weak activity in splitting the amide bond of glutamine and asparagine. When pyruvate is added, the desamidation is enormously increased without change in the final pyruvate concentration. This increase has been interpreted as being due essentially to two consecutive steps: (1) A condensation of the amide with pyruvate to form the dehydropeptide, followed by (2) the splitting of the dehydropeptide by dehydropeptidase in the tissues to form products which include pyruvate and ammonia. The increase in ammonia is accompanied by a corresponding decrease in amide nitrogen.

The dipeptide, pyruvoylglycine, does not exhibit this effect, and this fact has been interpreted on the basis either of its failure to condense with the amide or of its actual condensation with the amide to form a tripeptide in which the dehydroalanyl residue is in the middle of the chain and hence insusceptible to dehydropeptidase.

Extracts of the primary rat hepatoma do not show the pyruvate effect on amide desamidation to anywhere near the extent shown by extracts of normal liver, a phenomenon interpreted on the basis of the relative failure of the amide to condense with the keto acid in the former tissue.

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ORGANIC RADIOIODO COMPOUNDS FOR CANCER RESEARCH[1,2,3]

By HENRY S. BLOCH, *John Eisman Fellow 1944–46*, with FRANCIS EARL RAY, *Gastric Research Laboratory, Cincinnati General Hospital, and the Department of Chemistry, University of Cincinnati*[4]

INTRODUCTION

The two methods which have been used most successfully in the treatment of malignant neoplastic diseases are surgery and radiation (*1*). The success of the former depends primarily on diagnosis of the disease prior to the development of metastases. For X-ray or radium to be effective, conditions must be such that the neoplasm can be exposed to radiation of sufficient intensity to destroy the tumor without injury to neighboring healthy tissues and organs (*2*).

At this time, there is little hope for the discovery of a general diagnostic test for malignant neoplastic diseases which would be similar in effect to the Wassermann test for lues (*3*). Opinions vary whether cancer is one disease entity with subsidiary variations or a group of distinctly different diseases (*4*). But in studies dealing with possible methods for the diagnosis and treatment of neoplastic diseases it is best to treat each form of malignant growth as a separate malady (*5*).

Depending on the interpretation of statistics, the stomach may be considered the most common site of neoplastic growth in man, and gastric tumors are among the most difficult to control (*6*). While X-ray and gastroscopy have improved diagnoses of gastric disorders in general, neither could as yet be developed into a tool for the early diagnosis of gastric cancer. On the other hand, carcinoma of the stomach has a tendency to metastasize early, involving such vital parts of the body as the lymph nodes, liver, lungs, etc. These metastatic lesions are generally inoperable and not susceptible to the present methods of radiation therapy (*7*, *8*).

If radioactive compounds could be found that would localize with a certain degree of selectivity in or close to a malignant gastric neoplasm, they might well be useful in the diagnosis and treatment of carcinoma of the stomach. Administration of such a compound to a patient with gastric cancer would result in an accumulation of radioactive material at the site of the lesion. This localization of radioactive substance in the patient's stomach may be detected externally by placing photographic films or Geiger-Mueller tubes in suitable positions on the patient's chest, back, and sides. Since, however, the radioactive substances at present available for this work are chiefly short-range, beta-ray emitters, it is probably more advantageous to measure the radioactivity (*9*) in the stomach directly. This can be accomplished by fastening a piece of photographic film or a small Geiger-Mueller counter in place of the bucket of a Rehfuss tube or on the end of a gastroscope. These devices may then be introduced into the patient's stomach and the radioactivity measured in situ. It remains to find a radioactive substance that will localize selectively in a gastric neoplasm.

A number of organic compounds are known to localize with a high degree of selectivity in certain organs. Examples are the contrast media used in X-ray diagnoses for the visualization of the gall b'adder or urinary tract, such as sodium tetraiodophenolphthalein, sodium 2-oxo-5-iododihydropyridine-N-acetate (Iopax), sodium monoiodomethanesulfonate (Abrodil, Skiodan), and many others. Similarly, the concentration of sulfapyridine in the gastric juice has been studied by one of us and associates (*10*).

In 1941 Zahl and Cooper (*11*, *12*) injected lithium salts of various dyes such as trypan blue, Pontamin Sky Blue 6B, and carminic acid into tumor-bearing mice. These compounds were to be used in the therapy of cancer in conjunction with slow neutrons. Slow neutrons are biologically rather inert. However, impact of a slow neutron on a lithium atom leads to a nuclear reaction with a high release of energy. The particles thus formed have a very destructive influence on the tissues. The lithium salts of the dyes were supposed to localize selectively in the tumors, thereby producing a high concentration of lithium in the region where a maximum radiation effect was desired. As might have been expected, Zahl and Cooper found that the lithium salts of these dyes dissociated rapidly in the body, and that the lithium ions went their own way and did not deposit with the dye molecules.

Tobin and Moore (*13* and cf. *14*) were the first to report a synthesis of radioactive dyes. They prepared radioactive dibrom-trypan blue and radioactive dibrom-Evans blue by brominating *o*-tolidine with Br^{82} (half-life 34 h) and coupling the radioactive dibromo-*o*-tolidine with H-acid or Chicago acid, respectively. Moore's group investigated the distribution of these radiobrom dyes in tumor mice. Most of the radioactivity was found to be present in the liver. The ratio between the radioactivity in the various tumors

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and the livers of the animals was generally around 0.5, that is, the liver of an animal took up about twice as much radioactivity as did the tumor. While these findings cast some doubt on the selectivity of dye uptake by tumors, in the words of Moore and his co-workers—

> the significant fact remains, that the colloid permeates into and therefore radiates tumor tissue wherever this tissue may be, and no matter how widespread the metastases are (*15*).

Since many dyes become bound to proteins and stain carbohydrates "fast," and because the quantities used for injection have to be comparatively small, it is very difficult to analyze the tissues for their dye content chemically. Most observers have merely estimated the distribution of the dyes by macroscopic or microscopic inspection, and noted the intensity of the staining by a 1+, 2+, 3+, etc., designation. The reports in the literature and our own experience show that by this method agreement between macroscopic and microscopic findings is poor. Grossly, the presence of dye can be more readily seen in light-colored tissues or organs, such as the kidney cortex or tumor tissue, than in dark organs such as the liver or spleen. On studying the toxicity of iodo-trypan blue in dogs,[5] we found grossly that the cortex of the kidney was most intensely stained, while little discoloration was detectable in the spleen or liver. Under the microscope the picture was just about reversed. Granules of the dye were most frequently observed in the liver and spleen, where they were stored in the reticulo-endothelial elements (Kupffer cells), but rarely in the kidney. This discrepancy can be explained readily. The dye granules observed microscopically are due to an accumulation of the pigment by phagocytosis in form of small lumps; these lumps are then stored in the reticulo-endothelial system. They are readily visible under the microscope. The staining of the kidney, however, is evidently due to the presence of dye in a fine dispersion, similar to the dispersion in serum. In this form, the dye cannot be seen under the microscope at low concentrations. The work of Moore and Tobin showed clearly that determination of radioactive dyes in tissues with Geiger-Mueller counters is more accurate than the chemical and tinctorial assays which have heretofore been employed.

In view of the investigations just discussed, it appeared desirable to prepare more radioactive dyes and related compounds in the hope that they might become useful in the diagnosis and treatment of malignant neoplasms, particularly carcinoma of the stomach, or that they might at least help to elucidate some of the theoretical and practical problems involved.

[5] Bloch, H. S. Ph.D. thesis, University of Cincinnati. 1946.

Moore and Tobin chose Br^{82} to make their dyes radioactive; but this isotope has the rather short half-life of 34 h, and in addition it was difficult to obtain from the cyclotron. To make radioactive organic compounds more generally available for tumor research, it was desirable to employ an isotope with a somewhat longer half-life, which had a radiation suitable for biologic work and which could be synthesized into organic compounds with a firm linkage. I^{131} (half-life 8 d) met these specifications.

Our choice of compounds was influenced by the work on dyes reported previously and by our experience with sulfonamides (*10*). Hence we wanted to make available a number of radioiodo derivatives of mono- and bis-azo dyes, covering a range of molecular weights, solubilities, and degrees of acidity and basicity. Our attention was focused on compounds that would retain the iodine in the organic molecule despite metabolic processes. In developing the syntheses, we tried to take into consideration the practical needs of investigators who might wish to use these radioiodo compounds in physiologic studies. The 8-day half-life of I^{131} makes it necessary to prepare the radioactive compound shortly before it is used. Therefore we developed procedures for radioiodination which can be carried out quickly. The quantity by weight used in a physiologic study is generally well below 1 gm., and for this reason we studied some syntheses on a micro scale.

Ordinary iodo compounds were prepared as models for the corresponding radioiodo compounds. Iodination procedures were investigated primarily with regard to their adaptability to radioiodination. Thus, we were interested mainly in quantitative yields with respect to iodine. Three methods of iodination were employed: elementary iodine in alkaline medium, the Sandmeyer reaction, and iodine monochloride in acid medium.

4-Hydroxyazobenzene-4′-sulfonic acid (*16, p. 2192*) when treated with equimolar amounts of elementary iodine (I_2) in aqueous sodium hydroxide solution at 50°–100° C. took up only about one-tenth the amount of iodine expected theoretically for 4-hydroxy-3-iodoazobenzene-4′-sulfonic acid.[6] The procedure gave more complete iodination of azo dyes containing two hydroxy or amino groups in meta position to each other and an unsubstituted ortho-para carbon. About two-thirds of the amount of iodine expected theoretically for 2, 4-dihydroxy-5-iodoazobenzene-4′-sulfonic acid[7] and 2,4-diamino-5-iodoazobenzene-4′-sulfonic acid[8] could be introduced into 2, 4-dihydroxyazobenzene-4′-sulfonic acid (*16, p. 2195*) and 2,4-diaminoazobenzene-4′-sulfonic acid (*17, p. 2196*).

[6, 7, 8] See footnote 5.

The azo linkage rendered phenols and amines less susceptible to iodination, possibly due to increased resonance in the molecule. 4-Iodo-2-methylaniline could be prepared readily by refluxing a mixture of *o*-toluidine, iodine, calcium carbonate, water, and ether, followed by steam distillation (*18*, *19*).

The preparation of 1-hydroxy-8-iodo-3,6-naphthalenedisulfonic acid (*20*) (iodo-H-acid) from H-acid (1-hydroxy-8-amino-3,6-naphthalenedisulfonic acid) by the Sandmeyer reaction proved difficult.[9] Unless the reaction medium is kept at a pH of less than 2, diazo-H-acid has a strong tendency to couple with itself, forming a purple pigment which is very soluble in water. On decomposition of the diazonium compound, the amino group is replaced chiefly by a hydroxyl group. The 8-hydroxy and 8-iodo derivatives are both very soluble in water and difficult to separate. The 8-iodo compound was obtained by repeated precipitations from aqueous solutions with alcohol and ether. Iodo-H-acid was found to couple readily in alkaline solution with diazotized amines; its reaction with diazotized sulfapyridine gave a red compound (1-N^1-2''-pyridyl-4'-sulfamylphenylazo) - 1 - hydroxy-8-iodo-3,6-naphthalene-disulfonic acid),[10] and coupling with diazo-*o*-tolidine (*21*, *p. 145*) gave iodo-trypan blue (3,3'-dimethylbiphenyl-4,4'-bis (2-azo-1-hydroxy-8-iodo-3,6-naphthalenedisulfonic acid)).[11] The latter compound could also be prepared from trypan blue (*21*) (3,3'-dimethylbiphenyl-4,4'-bis(2-azo-1-hydroxy-8-amino-3,6-napthalenedisulfonic acid)) by the Sandmeyer reaction. Trypan blue could be diazotized readily with sodium nitrite in acid aqueous solution. When the diazonium compound was decomposed in the presence of iodide, the final product contained 70–80 percent of the amount of iodine expected theoretically for iodo-trypan blue.[12]

Sulfanilamide could be iodinated readily with iodine monochloride in acid aqueous solution by Scudi's method (*22*). The iodosulfanilamide so obtained could be diazotized and coupled. Iodination of sulfapyridine with iodine monochloride in acid aqueous medium gave monoiodosulfapyridine in good yields. Sulfathiazole seemed to decompose when treated with iodine monochloride.

Radioiodo Compounds

Radioiodine (I^{131}, half-life 8 d) was obtained from the Department of Physics, of the Massachusetts Institute of Technology. It was received as sodium radioiodide in 1–10-cc. aqueous solution, containing less than 1 mg. iodine by weight and activities of 100–2,000 microcuries. Ten to one hundred microcuries was used for a radioiodination experiment, affording a convenient range for activity assay on our instruments. Radioactivity measurements were carried out initially with a Lauritsen electroscope (*9*), and later on a commercial type Geiger-Mueller counter.[13] The organic radioiodo compounds were assayed in uniform 4-cc., screw-cap, glass vials, either in solution or as solids, and compared with suitable dilutions of the sodium radioiodide solution which had served as the source of radioiodide. A standard geometry was employed for each set of experiments.

Radioiodo-trypan blue was synthesized from trypan blue by the Sandmeyer reaction. The final product was a mixture of hydroxy-, iodo-, and radioiodo-trypan blue.

Since iodination with iodine monochloride had been so successful, an attempt was made to prepare radioiodine monochloride from sodium radioiodide by an exchange reaction. An aqueous solution of sodium radioiodide was shaken up with a solution of iodine monochloride in chloroform. An appreciable amount of radioactivity entered into the chloroform phase; however, under the conditions of the experiment the iodine monochloride decomposed.[14]

The desired result was achieved when it was found that radioiodosulfanilamide and radioiodosulfapyridine can be obtained by treating an acid aqueous solution of the parent sulfonamide and sodium radioiodide of the desired activity with iodine monochloride. The yields with respect to radioactivity are very satisfactory in this procedure. Radioiodosulfanilamide and radioiodosulfpyridine can be diazotized and coupled. Thus they give access to a great variety of radioiodo compounds.

EXPERIMENTAL PROCEDURE

Monoiodosulfanilamide (4-amino-3-Iodobenzenesulfonamide)

One-tenth mole sulfanilamide (17.2 gm.) was dissolved at 35° C. in 1,000 cc. water acidified with 30 cc. concentrated hydrochloric acid. The solution was cooled to 10° and 0.1 mole ICl (16.2 gm.) added under effective stirring. A brown precipitate formed. The mixture was left standing at room temperature for several hours; then the precipitate was filtered off. It was dissolved in 800 cc. water containing 25.2 gm. Na_2SO_3 (0.2 mole) at boiling temperature. The hot solution was filtered quickly by suction, leaving a small residue. On cooling, monoiodosulfanilamide crystallized from the mother liquor in long white needles. The crystals were filtered off and washed with water until the washings no longer decolorized a dilute

[9, 10, 11, 12] See footnote 5.

[13] Cyclotron Specialties Co., Moraga, Calif.

[14] See footnote 5.

aqueous iodine solution. They were dried in vacuo over P_2O_5. A sample of the compound dissolved in dilute nitric acid gave no precipitate with Ag^+ indicating absence of halogen ions. Yield: 15 gm.; 50 percent theory. Analysis (Parr bomb): Calculated for $C_6H_7O_2N_2SI$: I, 42.6; S, 10.7. Found: I, 43.5; S, 10.8.

MONOIODOSULFAPYRIDINE (4-AMINO-3-IODO-N^1 (2'-PYRIDYL)-BENZENESULFONAMIDE)

One-tenth mole sodium sulfapyridine.H_2O (28.9 gm.) was dissolved in 700 cc. water containing 35 cc. concentrated hydrochloric acid. The solution was cooled to 10° C. and 16.2 gm. ICl (0.1 mole) added under effective stirring. A beige precipitate formed presently. The mixture was stirred at room temperature for 1 hour; then the precipitate was filtered off. It was boiled with 2½ l. water containing 25.2 gm. Na_2SO_3 (0.2 mole); the sulfonamide remained largely undissolved. The hot mixture was filtered. The residue was dissolved in 100 cc. boiling acetic acid, 5 gm. Darco was added, and the solution filtered while hot. On cooling, large crystals separated from the brown filtrate. They were filtered off and recrystallized from 30 cc. glacial acetic acid. The final crop was washed with cold glacial acetic acid and water and then dried in vacuo over P_2O_5. The compound was found to be free of halogen ions. Yield: 8 gm.; 21 percent theory. Analysis (Parr bomb): Calculated for $C_{11}H_{10}O_2N_3SI$: I, 34.0; S, 8.5. Found: I, 34.5; S, 8.6.

MONOIODOSULFANILAMIDE-AZO-NEVILE-WINTHER ACID (2-(2'-IODO-4'-SULFAMYLPHENYLAZO)-1-HYDROXY-4-NAPHTHALENESULFONIC ACID)

A solution of 0.04 mole monoiodosulfanilamide (12 gm.), 4 gm. NaOH (0.1 mole) and 0.04 mole $NaNO_2$ (2.8 gm.) in 100 cc. water was prepared. This solution was dropped slowly (within one-fourth hour) into 400 cc. water containing 25 cc. concentrated hydrochloric acid (one-fourth mole HCl), under effective stirring. A fine, orange-yellow suspension formed. The temperature of the diazotization mixture was maintained at 0°–3° C. Stirring in the cold was continued for one-half hour, whereupon the mixture was ready for coupling.

A solution of 0.04 mole Nevile-Winther salt (sodium 1-hydroxy-4-napthalenesulfonate) (10 gm.), 16 gm. Na_2CO_3 (0.15 mole), and 1 gm. NaOH was prepared and cooled to 0°–3° C. The cold diazotization mixture was slowly added to this alkaline solution under effective stirring. A fine, cinnabar-red precipitate appeared. The mixture was stirred at room temperature for several hours and then left standing for a day. It was then warmed on a water bath, whence the precipitate dissolved. The solution was evaporated to about 200 cc., acidified with 20 cc. glacial acetic acid, and cooled in an ice bath. The wine-red precipitate thus obtained could be filtered readily. The residue was extracted with 95 percent ethyl alcohol at boiling temperature until the extracts failed to give a precipitate with a few drops of concentrated sulfuric acid, i. e., until the compound was free of Na^+. It was dried in vacuo over P_2O_5. Yield: 5 gm.; 23 percent theory. Analysis (Parr bomb): Calculated for $C_{16}H_{11}O_6N_3S_2INa$: I, 22.9; S, 11.5. Found: I, 22.9; S, 10.7.

MONOIODOSULFANILAMIDE-AZO-CHICAGO ACID (7-(2'-IODO-4'-SULFAMYLPHENYLAZO)1-AMINO-8-HYDROXY-2, 4-NAPHTHALENEDISULFONIC ACID)

Twelve grams of monoiodosulfanilamide (0.04 mole) was diazotized as described previously.

The diazotization mixture was added to a solution of 0.04 mole Chicago acid (1-amino-8-hydroxy-2,4-napthalenedisulfonic acid) (13 gm.), 18 gm. Na_2CO_3 (0.17 mole), and 1 gm. NaOH in 50 cc. water at 0°–3° C. under effective stirring. A thick, wine-red, colloidal solution formed. It was evaporated on a steam bath to about 80 cc. and acidified with 25 cc. glacial acetic acid, whence a chocolate-brown dye precipitated. The mixture was digested on a hot-water bath for a short time and then filtered while warm. The residue was suspended in a few cubic centimeters water, 3 volumes of 95 percent ethyl alcohol were added, and the mixture was left standing overnight at room temperature. The dye was then filtered off, washed with 75 percent ethyl alcohol, and dried in vacuo over P_2O_5. Yield: 2.5 gm.; 10 percent theory. Analysis (Parr bomb): Calculated for $C_{16}H_9O_9N_4S_3INa_4$: I, 17.8; S, 13.4. Found: I, 17.8; S, 13.1.

RADIOIODO-TRYPAN BLUE

One-third millimole of trypan blue (320 mg. tetra sodium salt) was dissolved in 5 cc. water. The dark purple-blue solution was acidified with 1 cc. 5M sulfuric acid (5 millimole H_2SO_4) and diazotized at 0°–5° C. by gradual addition of two-thirds millimole $NaNO_2$ (46 mg.) dissolved in 1 cc. water. The solution changed its color to green-gray. The diazotization mixture was stirred for one-half hour in the cold, then 100 mg. ammonium sulfamate was added to destroy any excess nitrite, and stirring was continued for another one-fourth hour. Then 1 cc. of radioiodide solution of the desired activity containing 15 mg. sodium iodide (0.1 millimole) was added. The solution was slowly warmed to 50° C. and stirred at that temperature for 6 hours. The color changed to purple-red. Finally the solution was heated to 96°–100° C. and kept at that temperature for another hour. After it had cooled to room temperature, the dye was precipitated with

50 cc. ethyl alcohol and 100 cc. peroxide free ethyl ether and separated from the mother liquor by centrifuging. The compound was then dried, taken up in a suitable amount of water, and the pH was adjusted to 7.0–7.5 with sodium hydroxide and sodium bicarbonate solutions. A glass electrode was used to check the pH values. For injection, the dye solution was made up to the desired concentration with physiologic saline, filtered, and sterilized.[15] The yields with respect to radioactivity were around 70 percent.

Monoradioiodosulfanilamide (4-amino-3-radioiodobenzenesulfonamide

To 8.5 gm. sulfanilamide (0.05 mole) were added 15 cc. concentrated hydrochloric acid and then 500 cc. water. On stirring at room temperature all the sulfonamide dissolved. The solution was cooled to 10° C. by addition of a few pieces of ice. Twenty-five cubic centimeters of sodium radioiodide solution was added, and then under effective stirring 8 gm. iodine monochloride (0.05 mole); a beige precipitate appeared. The mixture was stirred at room temperature for 1 hour and then left standing in the laboratory for 2 days. The beige-brown precipitate settled; the supernatant liquid was clear and slightly yellow. The precipitate was filtered off, washed with water and then with ethyl ether, and dried in vacuo over P_2O_5. (1) Yield: 11 gm. monoradioiodosulfanilamide (beige, amorphous); 74 percent theory by weight.

Nine grams of the crude monoradioiodosulfanilamide was dissolved at boiling temperature in 600 cc. water acidified with 2 cc. 6N hydrochloric acid and containing 10.4 gm. $NaHSO_3$ (0.1 mole), giving a yellow solution. The hot solution was filtered quickly by suction (leaving no residue) and then cooled at about 3° C. for 3 hours. A white, crystalline precipitate separated. It was filtered off, washed with water until the washings no longer decolorized a dilute aqueous iodine solution, and dried in vacuo over P_2O_5. (2) Yield: 7 gm. monoradioiodosulfanilamide (crystalline, white); 64 percent by weight of (1), 47 percent by weight of sulfanilamide.

Radioactivity measurements were made on the Geiger-Mueller counter (scaling circuit of 1:8). The samples (about 1 gm.) were placed in 4-cc., screw-cap, glass vials, and the vials were put under the bell-type counter tube.

Results of activity measurements in counts as recorded on the instrument (all measurements made on same day):

Counts added (in 25 cc. NaI* solution, containing 0.0025 millimole NaI) :2,450 C./minute.

Specific activity of crude monoradioiodosulfanilamide: 184 C./minute/gram.

Total activity in 11 gm. crude monoradioiodosulfanilamide (1) : 2,024 C./minute.

Percent of counts recovered for crude monoradioiodosulfanilamide: 82.

Specific activity of recrystallized monoradioiodosulfanilamide: 205 C./minute/gram.

Total activity in 7 gm. recrystallized monoradioiodosulfanilamide (2) : 1,435 C./minute.

Percent counts recovered for recrystallized monoradioiodosulfanilamide: 58.

Approximate specific activity of recrystallized monoradioiodosulfanilamide: 1 microcurie/gram.

Monoradioiodosulfapyridine (4-amino-3-radioiodo-N[1] (2′-pyridyl)-benzenesulfonamide)

One-tenth mole sodium sulfapyridine.H_2O (28.9 gm.) was dissolved in 1,000 cc. water containing 35 cc. concentrated hydrochloric acid. The solution was cooled to 10° C. by addition of a few pieces of ice. Fifty cubic centimeters of sodium radioiodide solution was added, and then under effective stirring 16 gm. iodine monochloride (0.1 mole). A beige precipitate formed. The mixture was stirred at room temperature for 1 hour. Then the precipitate was filtered off. The filtrate was greenish yellow, and a small amount of a white precipitate separated after a while. The residue was washed with water and dried in vacuo over P_2O_5. (1) Yield: 38 gm. monoradioiodosulfapyridine, crude (dark beige, amorphous); 100 percent by weight.

Thirty-four grams of the crude compound was suspended in 1,000 cc. water containing 21 gm. $NaHSO_3$ (0.2 mole) and 10 cc. 6N hydrochloric acid and was heated to boiling under stirring. The precipitate remained largely undissolved. The mixture was cooled in an ice bath. Then the residue was filtered off and washed with water until the washings no longer decolorized a dilute aqueous iodine solution, and finally with ether. The compound was dried in vacuo over P_2O_5. (2) Yield: 31 gm. monoradioiodosulfapyridine (light ocher, amorphous); 91 percent by weight.

Radioactivity measurements were carried out as described previously. Results (all measurements made on same day):

Counts added (in 50-cc. NaI* solution, containing less than 0.001 mg. I); 4,875 C./minute.

Specifc activity of crude monoradioiodosulfapyridine: 100 C./minute/gram.

Total activity in 38 gm. crude monoradioiodosulfapyridine (1) : 3, 800 C./minute.

Total activity in 31 gm. purified monoradioiodosulfapyridine (2) : 3,131 C./minute.

Percent counts recovered for purified monoradioiodosulfapyridine: 64.

[15] See footnote 5.

Approximate specific activity of recrystallized monoradioiodosulfapyridine; 1 microcurie/gram.

NOTE: To obtain good yields it is essential that the iodination be carried out in a dilute solution and that the iodine monochloride be added slowly and under effective stirring.

SUMMARY

The use of radioactive organic compounds which localize selectively in tumors is discussed. Possible methods for the diagnosis and treatment of malignant neoplasms, particularly carcinoma of the stomach, are suggested. A variety of radioactive dyes, dye intermediates, and sulfonamides, which may show selective localization in tumors, was obtained by incorporating radioiodine into aromatic amines and phenols. Ordinary iodo derivatives of promising compounds were prepared. They served as models for the corresponding radioiodo compounds.

The synthesis of radioiodo derivatives from radioiodide is described. Radioiodo-trypan blue was synthesized from trypan blue by the Sandmeyer reaction. Radioiodosulfanilamide and radioiodosulfapyridine were obtained in good yields with respect to radioiodine by treating an acid aqueous solution of the parent sulfonamide and sodium radioiodide with iodine monochloride.

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EFFECT OF ADMINISTRATION OF HOT WATER, ACIDS, ALKALI, MECHOLYL CHLORIDE, OR ATROPINE SULFATE UPON THE GASTRIC MUCOSA OF MICE[1]

By HELEN M. DYER, *biochemist*, MARGARET G. KELLY, *assistant biochemist*, and THELMA B. DUNN, *research fellow in pathology, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The papers (*1*) that were presented at a conference on gastric cancer[2] in 1940, supplemented by those (*2*) from a conference[3] held in 1944, cover the literature adequately on both clinical and experimental gastric cancer. The high incidence of gastric cancer in man, its diagnosis, treatment, etiology, and the question of a genetic influence were discussed in detail. No attempt is made at this time, therefore, to review the literature.

Voegtlin (*3*) emphasized the value of the production of a disease under well-defined experimental conditions as an important contribution to an understanding of the process of the disease. At the present time, no confirmed report has been found of the production of gastric adenocarcinoma in animals with the oral administration of any agent. Stewart (*4*) induced neoplasms of the forestomach of mice with the oral administration of methylcholanthrene or 1, 2, 5, 6-dibenzanthracene. Stewart and Lorenz (*5*) succeeded in producing gastric adenocarcinoma in several strains of mice by the injection of emulsions of these two carcinogens directly into the wall of the glandular portion of the stomach. Production of adenocarcinomas in the stomachs of mice by a combination of selective breeding and parenteral injections of methylcholanthrene was recently reported by Strong (*6*).

The etiology of gastric cancer, however, is not known. The present investigation was undertaken with the hope of producing cancers or precancerous lesions in the gastric mucosa of mice by the repeated administration of tissue irritants and of agents which had been shown to modify the secretion of gastric juice. In addition to this treatment, some groups of animals were given an emulsion of 1, 2, 5, 6-dibenzanthracene repeatedly by esophageal intubation (*7*).

The various agents failed to produce chronic gastric lesions under the conditions of the experiment. The report is therefore presented with a minimum of detail.

MATERIALS AND EXPERIMENTAL PROCEDURE

Inbred mice of strains A, C3H, and I, and hybrid LA mice (strain L mice crossed with mice of strain A) of the F_1, F_2, and F_3 generations were selected for the experiment. Both males and females were used, except in the case of mice of strain C3H, of which only males were selected because of the high incidence of spontaneous mammary tumors in the females. The mice were separated according to sex and were housed in metal, raised-bottom cages in groups of five.

Pellets similar in composition to diet 77 (*8*) were fed ad libitum, except on treatment days when the food was removed from all cages approximately 4 hours before the injections and was returned within the hour following treatment. When the material was administered in the drinking water, the food was removed from these cages also for the same periods as those in which the injected mice fasted. Water in bottles with special outlet tubes was available at all times.

The agents administered were hot distilled water, hydrochloric acid, lactic acid, sodium hydroxide, mecholyl chloride (acetyl-β-methylcholine chloride), and atropine sulfate. Groups of mice from each of the strains were evenly distributed with regard to age and sex and then subjected to treatment with each agent. The mice from those groups that also received repeated injections of an emulsion of 1,2,5,6-dibenzanthracene were matched with mice that received a control emulsion of mineral oil and sodium alginate supplementary to the specific agent.

The carcinogenic hydrocarbon was emulsified by the gradual addition of a solution of 1,2,5,6-dibenzanthracene in hot mineral oil to a hot aqueous solution of sodium alginate. A mechanical stirrer was used during the addition of the hydrocarbon solution, and the dissolved alginate was kept in a water bath at a temperature below boiling. The final emulsion contained, per cubic centimeter, approximately 2 mg. of dibenzanthracene and 8 mg. of sodium alginate.

During the course of the treatments, the mice suffered such a loss of appetite and of body weight that the regimen as originally adopted had to be changed. The dosage was decreased; the periods of treatment were shortened; the intervals between treatments were increased; and whenever the majority of the mice showed loss of weight or other unfavorable symptoms, the injections were discontinued temporarily. During the first 5 months of the experiment injections were made 5 times weekly from Monday through Friday, except those with mecholyl and atropine sulfate, which were administered 10 times weekly (twice a day on each of the 5 days). The dibenzanthracene

[1] Received for publication August 23, 1946.

[2] Under the auspices of the National Advisory Cancer Council, held at the National Institute of Health, Bethesda, Md., on October 11 and 12, 1940.

[3] Under the auspices of the Committee on Cooperation in Cancer Research, National Advisory Cancer Council, United States Public Health Service, held at The Memorial Hospital for the Treatment of Cancer and Allied Diseases, New York City, on November 11, 1944.

and control emulsions were begun later. At this early period, because of the great mortality among the treated mice, additional animals were started in each group, and the number of injections was decreased to 3 a week on alternate days. When the dibenzanthracene and control emulsions were given during a later period, they were administered once a week. Lactic acid and atropine sulfate were furnished continuously in the drinking water except during short periods when great loss of weight was observed and the mice were given drinking water without the supplements.

For the treatment with hot water care was taken to cool the needle by immersion in cold water before inserting it in the mouth of the animal.

The effect of subcutaneous injections of mecholyl into mice upon the secretion of gastric juice was investigated before the repeated injections were begun. The procedure described by Roe and Dyer (*9*) was followed except that urethane was used to anesthetize the mice and the peptic activity was measured by a modification (*10*) of the method of Anson. An attempt was made to prolong the action of mecholyl chloride by incorporating it into a beeswax-liquid petrolatum emulsion and injecting it intramuscularly in amounts up to 2.0 mg. of mecholyl per mouse. Profuse salivation and bulging of the eyes and occasional gastric hemorrhages resulted, but the action of the mecholyl was not prolonged significantly. After three such injections into the experimental mice, the subcutaneous injections of aqueous solutions of the gastric stimulant were resumed.

After 2 months of subcutaneous administration of atropine sulfate, intramuscular injections of a beeswax-liquid petrolatum emulsion of this substance were tried. Abscesses appeared at the sites of injection, so that the subcutaneous injections of the aqueous solution were resumed after 10 days. When the number of injections of the various agents was reduced to three a week, the surviving mice in this group and the new mice added to the group were given atropine sulfate in their drinking water instead of subcutaneously.

Preliminary tests with esophageal intubations of the alginate-mineral oil emulsion to mice that were killed at intervals of 0.5, 2.0, and 6.0 hours following the injection demonstrated the persistence of a white gel in the stomach. The gel adhered to the gastric mucosa and frequently appeared to form a plug in the pyloric region of the stomach. Supplementary injections of hydrochloric acid or of hot water had no observable effect upon the persistence of the emulsion in the stomach; sodium hydroxide changed the white gel of the injected emulsion to a yellowish, mucilaginous fluid which was found to remain in the stomach for some hours.

The loss of mice was so great during the first year of the investigation that it was decided thereafter to discontinue treatment of all mice that had been treated for as long as 6 months (65 to 75 injections) and to keep the surviving animals under observation until they began to show signs of abnormality, or loss of weight, or had reached the age of 12 to 15 months.

The dosages of the agents and the numbers of injections are summarized in table 1.

TABLE 1.—*Summary of treatment of mice*[1]

Agent	Concentration[2]	Number of injections per mouse[3]		Number of mice		Age of Mice at—				Final weights of mice		Appearance of stomachs at necropsy			
						First treatment		Necropsy				Gross		Microscopic	
		Range	Average	Died	Killed	Range	Average	Range	Average	Range	Average	Normal	Hemorrhagic	Normal	Pathologic[4]
						Months	*Months*	*Months*	*Months*	*Grams*	*Grams*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Water, approximately 60° C.		15–141	57	34	28	1–11	4	3–22	11	11–41	23	92	8	93	0
Control emulsion		4–23	13			1–13	6								
Water, approximately 60° C., and.		11–129	67	14	18	1–6	3	3–17	11	10–36	24	94	6	72	0
Dibenzanthracene emulsion.	2 mg. per cc.	3–16	11			1–8	5								
Water, room temperature.		16–131	62	20	14	1–8	4	7–18	12	17–34	26	93	7	93	7
Control emulsion		3–23	13			1–10	5								
Water, room temperature, and.		27–138	75	15	17	1–6	2	5–18	11	15–46	29	97	3	82	12
Dibenzanthracene emulsion.	2 mg. per cc.	2–17	13			1–6	3								
Hydrochloric acid	1.0 to 0.25N	10–92	41	35	23	1–7	3	2–18	9	10–45	24	88	12	65	4
Control emulsion		3–21	12			1–10	4								
Hydrochloric acid, and	1.0 to 0.25N	19–99	54	18	22	1–7	3	4–17	11	13–52	25	93	7	86	0
Dibenzanthracene emulsion.	2 mg. per cc.	2–24	12			1–8	3								

See footnotes at end of table.

TABLE 1.—*Summary of treatment of mice*[1]—Continued

Agent	Concentration [2]	Number of injections per mouse [3]		Number of mice		Age of Mice at—				Final weights of mice		Appearance of stomachs at necropsy			
						First treatment		Necropsy				Gross		Microscopic	
		Range	Average	Died	Killed	Range	Average	Range	Average	Range	Average	Normal	Hemorrhagic	Normal	Pathologic [4]
						Months	*Months*	*Months*	*Months*	*Grams*	*Grams*	*Per cent*	*Per cent*	*Per cent*	*Per cent*
Sodium hydroxide	0.5 to 0.1N	20–132	51	19	17	1–8	3	3–18	10	17–37	28	90	10	70	12
Control emulsion		1–24	13			1–10	4								
Sodium hydroxide, and	0.5 to 0.1N	12–65	38	23	11	1–8	3	4–19	9	14–42	24	89	11	82	9
Dibenzanthracene emulsion.	2 mg. per cc	1–16	10			1–6	4								
Mecholyl chloride	0.05 to 0.5 mg. per cc.	56–306	151	19	15	1–5	2	5–17	9	13–38	25	91	9	73	0
Control emulsion		1–22	12			1–5	4								
Mecholyl chloride, and	0.05 to 0.5 mg. per cc.	62–286	168	15	11	1–5	2	5–15	11	17–37	26	89	11	91	0
Dibenzanthracene emulsion.	2 mg. per cc	3–22	13			2–5	4								
Atropine sulfate, s. c., or	0.1 to 1.0 mg. per cc	50–266	216	23	12	1–10	2	2–17	9	15–43	26	83	17	92	0
Atropine sulfate in drinking water.	0.25 mg. per cc	21–100	69			1–10	5								
Control emulsion		4–18	11												
Atropine sulfate, s. c., or	0.1 to 1.0 mg. per cc.	25–265	209	17	14	1–7	2	6–20	11	15–40	25	87	13	100	0
Atropine sulfate in drinking water, and.	0.25 mg. per cc	53–100	86												
Dibenzanthracene emulsion.	2 mg. per cc	5–17	12			1–7	4								
Lactic acid	0.2 to 0.1N	31–153	91	18	17	1–9	3	8–16	13	15–38	25	91	9	82	12
Control emulsion		7–28	15			1–9	4								
Lactic acid, and	0.2 to 0.1N	45–160	93	5	13	1–6	3	5–15	11	13–37	24	78	22	100	0
Dibenzanthracene emulsion.	2 mg. per cc	8–32	14			1–6	4								

[1] Mecholyl chloride was administered subcutaneously; atropine sulfate, subcutaneously and in drinking water; lactic acid in drinking water; and all other agents by esophageal intubation.

[2] The volumes administered were 0.01 cc. of agent per gram of body weight for all esophageal intubations and subcutaneous injections, and ad libitum when agent was administered in the drinking water.

[3] When agent was administered in drinking water the values are for days of treatment per mouse.

[4] Miscellaneous pathologic changes other than typical lesions in mice of strain I.

The mice were weighed biweekly during the period of treatment and once a month between the last injection and the time of killing. Many of the mice died during the night, and post mortem changes were too advanced for necropsy. Where post mortem changes were not too great, gross examination was made of each animal that died, special care being exercised in the examination of the gastrointestinal tract. The remaining mice were killed as stated previously. They were necropsied, and histologic studies were made.

In the routine examination of mice that were killed at an early date, the stomach was removed at once, quickly examined for gross lesions, cut into three longitudinal strips, and fixed in absolute alcohol for Mayer's mucicarmine stain, Regaud's solution for Bowie's pepsinogen stain, and Zenker-formol for routine hematoxylin-eosin stain. The rest of the organs were then examined, and those showing gross abnormalities were taken for routine hematoxylin-eosin stain. Later in the experiment, special stains on the stomach were usually omitted, but the routine hematoxylin-eosin stain was made of the stomachs of all the experimental mice that were killed, and the special stains and the hematoxylin-eosin stains were used for histologic studies of the stomachs of 15 untreated control mice of strains A, C3H, and I, and LA hybrids.

RESULTS

Table 1 summarizes the experiments and the findings with regard to the stomachs of all the mice that were necropsied. In the case of the animals that died during treatment, the only abnormality observed in any of the stomachs of any of the groups was hemorrhage. Petechial hemorrhages when present were invariably located in the glandular portion of the gastric mucosa and extended over a very small area. From preliminary studies of the acute toxic effects of the various agents on mice, it seems likely that the stomach hemorrhages were caused by the acute, irritating effects of the agents injected. Hemorrahages were observed in small areas of the intestinal tracts of 9 percent of the treated mice and were found in all groups.

No significant difference in the amount of mucin

or pepsinogen in the gastric mucosa could be detected in any of the groups of experimental or control animals, although occasional mice showed a considerable variation for which no cause was found. Positive mucin staining is found characteristically in a few glands near the limiting ridge (vestiges of a cardiac portion in the mouse), on the surface of the gastric gland or fundic portion, and in cells located at the base of the pyloric glands. The mucicarmine stain did not outline so distinct a layer of mucous cells at the necks of gastric glands as is usually encountered in the rat. Stomachs of strain I mice were of particular interest, since these special staining procedures disclosed a disarrangement of cell types and showed that mucous staining cells were present in the fundic glands before the typical strain I lesion, as described by Stewart and Andervont (*11*), had fully developed.

Except for those of the strain I animals, the stomachs examined were remarkably normal. A few miscellaneous pathologic changes were recorded which did not appear significant. Only 1 mouse, a hybrid LA female, which had received 92 injections of hydrochloric acid and 11 injections of dibenzanthracene over a period of 6.5 months and was killed at the age of 8.5 months, showed an inflammatory reaction about the base of the glands, and some hyperplastic cells. Another hybrid animal treated with atropine sulfate and dibenzanthracene developed an epidermoid carcinoma of the forestomach. Stomachs of the strain I animals showed the changes typical for this strain, which did not seem to be modified by treatment.

Numerous pathologic changes were found in other organs examined; but none of them could be ascribed to any specific experimental procedure, since they occur commonly in any groups of old experimental mice. Lesions in the lungs were very frequent, especially in animals subjected to esophageal injections. These pulmonary changes usually consisted of areas of consolidation caused by a leukocytic exudate or accumulations of large vacuolated cells in the alveoli, bronchitis, peribronchial leukocytic infiltrations, and areas of atelectasis. These pulmonary conditions were probably the most frequent immediate cause of death.

Several types of tumors were found, but they were not significantly more frequent in any particular group. Among them were mammary tumors, leukemias, and lung tumors. Nine of fourteen mice that developed tumors had received dibenzanthracene.

SUMMARY

Repeated direct alimentary administration of hot water, hydrochloric acid, sodium hydroxide, lactic acid, or atropine sulfate, with or without an emulsion of 1, 2, 5, 6-dibenzanthracene, failed to produce cancer or precancerous lesions in the gastric mucosa of mice of strains A, I, and C3H, and in hybrid LA mice (strain L crossed with mice of strain A).

Repeated parenteral injections of mecholyl chloride or of atropine sulfate, with and without direct alimentary administration of 1, 2, 5, 6-dibenzanthracene, were likewise ineffective in producing any specific pathologic changes in the gastric mucosa of mice.

That the stomachs showed so little injury following very drastic treatment is evidence of the remarkable resistance and recuperative power of the normal gastric mucosa.

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MAMMARY TUMORS IN MALE MICE IMPLANTED WITH ESTROGEN-CHOLESTEROL PELLETS[1]

By MICHAEL B. SHIMKIN, *surgeon*, and ROSE S. WYMAN, *laboratory technician, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

A recent review (*1*) on hormones and mammary cancer in mice concluded that data in the literature are insufficient regarding the relationship between dose of estrogen and the mammary-tumor response. In general, the incidence of mammary tumors is increased and the average time of appearance of the gross tumors is shortened with increase in the dose of estrogen until the toxic level is reached.

Studies with estrogens in oily or aqueous vehicles appeared to indicate that doses considerably larger than those that could be expected to be produced physiologically were necessary to elicit mammary tumors in male mice. Estrogens in oily or aqueous vehicles, however, have relatively short periods of action. With the introduction of methods of administration of estrogens in the form of solid pellets, allowing constant, protracted absorption of the chemicals, it was shown that the dose of estrogen needed to elicit mammary tumors is in the same dose range as that elaborated physiologically by normal female mice (*1*).

The use of estrogens in the form of subcutaneously implanted pellets has wide experimental applicability. Single injections, rather than tedious, repeated injections needed when the materials are dissolved in oil, simplify the work of the investigator. More important, repeated injections of oil over protracted periods are not well tolerated by mice. The dose and the absorption rate of the hormones can be adjusted by melting the estrogen in variable proportions with cholesterol and preparing fused pellets of desired size and concentration. It has been shown that the dose absorbed from solid pellets implanted subcutaneously is a function of the surface area of such pellets (*2*). Furthermore, the pellets can be removed from the animal, terminating the effect of the hormone, and the actual dose used can be determined with accuracy by weighing or otherwise assaying the amount of the chemical recovered.

The present communication reports results obtained in mice implanted with estrogen-cholesterol pellets. The experiment was designed to furnish information regarding the quantitative expressions of the relationship between the dose of estrogen and the mammary-tumor response.

EXPERIMENTAL PROCEDURE

The mice employed were males of the C3H strain (Andervont subline), born and raised at the National Cancer Institute. They were given Purina dog chow pellets as food and had an unlimited supply of water. They were kept in wooden boxes with wood shavings as bedding, five to eight animals to a box.

The estrogen-cholesterol pellets were prepared according to a technique described previously (*3*). The desired amounts of estrogen and of cholesterol were weighed, mixed, and melted in an oil bath. The liquid mixture was drawn into thick-walled glass tubes, 6 to 8 cm. in length, with a 1-mm. bore, by means of a well-fitting metal plunger. No lubricant was used in the tubes. As soon as the material solidified in the tube, it was extruded by the plunger. The solid rod of hormone-cholesterol mixture thus formed was divided by means of a razor blade into pellets 2 mm. long. The pellets were washed rapidly in 95 percent alcohol to smooth the rough ends.

In this manner were obtained pellets of cholesterol containing 0.5 to 30.0 percent of diethylstilbestrol, 1.0 to 20.0 percent of estradiol, and 10 to 50 percent of triphenylethylene.[2] The pellets were 1 mm. in diameter and approximately 2 mm. long. They weighed 2.0 mg. on the average; the extreme range of weights was between 1.5 and 2.5 mg., the great majority falling within the 1.8-to-2.2-mg. range.

The pellets were introduced into the subcutaneous tissue of the left axilla of mice by means of a small trocar. The animals were implanted with the hormone pellets when they were 1 month old and averaged 16 gm. in weight. Each mouse received one pellet.

The first group of animals was injected in October 1943, and groups distributed among the different estrogens and dose levels were added until November 1944. A total of 486 mice was used; of these, 25 were discarded from the results because of early deaths, faulty individual identification, or the failure to recover the hormone pellet at necropsy.

The mice were examined by palpation at weekly intervals. The latent periods of the tumors were determined by dates upon which definite subcutaneous nodules were first felt. Tumors and other tissues that in the gross appeared abnormal were examined under the microscope after appropriate fixation, section, and staining. Four large, rapidly growing, subcutaneous tumors were devoured by other mice or were too decomposed for sections

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[2] We are indebted to Merck & Co., Rahway, N. J., for the supply of diethylstilbestrol and triphenylethylene, and to Schering Corporation, Bloomfield, N. J., for the supply of estradiol. Cholesterol was obtained from Eastman Kodak Co., Rochester, N. Y.: it is a product of the spinal cord of cattle and has a melting point of 149°-150° C.

following the death of the animals, and were accepted as mammary tumors in the results on the basis of gross findings.

RESULTS

The results relative to the appearance of mammary tumors are presented in table 1. There is correlation between the concentration of diethylstilbestrol and of estradiol in the pellets and the incidence of mammary tumors, as well as the latent periods of appearance of the tumors. The data on pellets of diethylstilbestrol containing 2.0 and 5.0 percent of the estrogen appear to be reversed both as to incidence of tumors and the average latent time. The pellets used for these groups were assayed chemically (*3*) for the content of diethylstilbestrol at the termination of the experiment. No error was detected, and the discrepant results are attributed to chance variation. The results with the higher doses of estradiol were complicated by early mortality due to toxic effects of the hormone, similar to those described for diethylstilbestrol (*4*). No mammary tumors were obtained in mice receiving pellets containing triphenylethylene.

It appears that estradiol is more potent than diethylstilbestrol in eliciting mammary tumors in strain C3H male mice, at least until the toxic level is reached. Triphenylethylene is obviously much less potent than either diethylstilbestrol or estradiol.

The results with diethylstilbestrol pellets are in agreement with previous experiments in which similar, although larger pellets were used. Thus, with 10 percent diethylstilbestrol-in-cholesterol pellets weighing 6–8 mg., 10 mammary tumors were elicited in 22 strain C3H male mice, or in 45.4 percent; 9 mice all developed mammary tumors following the implantation of 25-percent pellets weighing 4–6 mg. (*1*).

The pellets were recovered at necropsy in all but one mouse, which is not included in the results. Some of the pellets were dried and weighed after the removal of adherent tissue. The greatest reduction in weight, 20 percent, occurred in a few pellets containing 30 percent of diethylstilbestrol. Other pellets showed an average reduction of 0.2 mg., or 10 percent, in weight.

It is therefore calculated that the effective dose for the induction of mammary tumors in C3H male mice was about 10 percent of the amount implanted in the pellets. Since the 2-mg. pellets containing 1 percent of diethylstilbestrol and of estradiol originally contained 0.02 mg. of the estrogens, it is estimated that only 2 micrograms of the chemicals given in this form was sufficient to produce mammary tumors in 5 percent of the animals. Previous determinations (*3*) established that the hormones are not selectively absorbed from the pellets.

With estradiol, a computed absorption of 4 to 10 micrograms of the hormone was sufficient to produce mammary tumors in approximately 50 percent of the animals. The 50-percent tumor dose (TD_{50}) with diethylstilbestrol was between 20 and 40 micrograms.

The dose of diethylstilbestrol in oil that produces vaginal cornification in female mice is 0.05 to 0.1 micrograms. The urinary excretion of estrogens in normal female mice is given as 0.05 to 0.1 micrograms per day (*1*). Thus, the amount of estradiol or diethylstilbestrol required to produce mammary tumors in about 50 percent of male mice of the C3H strain is roughly equivalent to the urinary excretion of estrogens in female mice during 100 or 300 days, respectively. The carcinogenic dose of estrogens for male mice, therefore, is well within the range of concentrations elaborated physiologically by normal females of the species.

Four mammary tumors appeared within 3 months after the implantation of estrogens. The earliest was found grossly 56 days after the mouse received a pellet containing 10 percent of estradiol, and another tumor in the same group appeared in 88 days. Two tumors were detected 75 days after implantation of 20- and 30-percent pellets of diethylstilbestrol.

Subcutaneous tumors arose in 150 mice of the experiment. The masses in 146 mice were examined under the microscope and were found to be mammary tumors. All these tumors were the usual adenocarcinomas of variable types usually encountered in mice (*5*). Four mice were accepted in the results as having mammary tumors on the basis of diagnoses in the gross.

There was no predilection for the occurrence of mammary tumors near the site of the implantation of the estrogen pellets. Of 155 tumors whose location was recorded, 47 appeared in the left anterior breasts of the animals, and 51 arose in the right anterior breasts; of 57 tumors that occurred in posterior breasts, 31 were on the left and 26 on the right side. There appeared to be correlation, however, between the occurrence of multiple mammary tumors and the larger doses of estrogen. Thirty-five mice developed more than 1 mammary tumor; of these, 10 had 3 tumors each, 1 had 4 tumors, and 1 animal developed 8 mammary tumors. Only 4 multiple tumors developed in animals that received pellets containing 5.0 percent or less of diethylstilbestrol or estradiol, or 8 percent of the 48 animals that developed tumors. Of the mice receiving pellets containing 10.0 percent or more of the 2 estrogens, 31 had more than 1 mammary tumor, or 30 percent of the 101 animals that developed tumors. All animals that developed 3 or more mammary tumors received pellets containing 10.0 or more

TABLE 1.—*Mammary tumors in male C3H mice implanted with estrogen-cholesterol pellets*

[Numerator=mice with mammary tumors
Denominator=mice dying without tumors]

DIETHYLSTILBESTROL

Concentration of estrogen in pellet (percent)	Estrogen in pellet	Appearance of mammary tumors and death of animals without tumors, in months after implantation of pellets																								Total mice (uncorrected)	Total tumors	Percentage of tumors	Average latent time
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26				
	Mg.																											*Number*	*Number*		*Months*
0.5	0.01									1/2	0/1		0/3	0/1	0/1	0/1		0/2	0/3	0/3	0/10	0/2	0/4	0/3	0/3			40	1	2.5	9.6
1.0	.02											0/2		0/1	0/1	0/4	1/2		1/1	0/5	0/7	0/1	0/4	0/2	0/2	0/1		35	2	5.7	16.8
2.0	1							1/0	1/0		3/0	1/3		0/3				0/2	0/5	0/1	0/1	0/2	1/4		0/2			30	7	23.3	11.5
5.0	.04						1/0			0/1	0/2	0/2	0/2		0/1	0/2		1/4	0/1	0/1		0/1	0/3	0/2	1/2		0/3	30	3	10.0	15.9
10.0	.2					2/1	2/2	0/4	2/0	2/0			1/2	0/4	0/1	1/0	0/1	0/1		1/0	0/1			0/2					30	11	36.6	9.8
20.0	.4		1/0		1/1	3/0	7/3	2/0	1/0	3/1	2/0		2/1			1/0			0/1	0/1	1/0							32	24	75.0	8.4
30.0	.6		1/0		3/0	7/0	6/2	2/1	2/0	1/0	4/0	0/1																30	26	86.6	6.9

ESTRADIOL

1.0	0.02				1/0	0/1	0/1			1/1			0/4	0/2	0/2	0/6		0/4	0/1		0/11	0/1	0/1	0/1	0/2			40	2	5.0	7.0
2.0	.04				1/0	2/0		3/1	1/0	1/0		0/1	0/1	2/0	0/2	0/2		0/1	0/3		1/4	0/1	0/2	0/1		0/1		31	11	35.5	9.2
5.0	.1				4/0	4/0	2/0	5/0	1/0		1/0	1/0	1/1	2/1	1/1			0/1	0/1		0/1					6/2		30	22	73.3	7.9
10.0	.2	1/0	1/0		3/0	8/0	4/0	4/0	1/1	1/0	0/1				0/1	0/1					0/1			0/1				29	23	75.8	5.9
20.0	.4			0/3	6/1	4/2	2/0	5/0	0/1																0/1			25	17	[1] 77.3	5.9

TRIPHENYLETHYLENE

10	0.2							0/1	0/1			0/1	0/1	0/1	0/1		0/2	0/1	0/1	0/1		0/1	0/3	0/3	0/3	0/3	0/2	26	0		
25	.5													0/2			0/4		0/1	0/1	0/1	0/1	0/5	0/1	0/6	0/2	0/3	27	0		
50	1.0										0/1			0/2	0/2	0/2	0/1	0/1		0/2	0/1	0/2	0/3	0/4	0/3		0/2	26	0		

[1] Not counting 3 mice dying before appearance of first tumor.

percent of the estrogens. The mice that were injected with pellets containing 10.0 or more percent of the estrogens uniformly exhibited brown degeneration of the adrenals and enlarged pituitary glands.

One subcutaneous mass, on the right flank (opposite to the side of the estrogen pellet), was found to be a spindle-cell sarcoma with areas of calcification. This tumor arose in a 21-month-old mouse of the group injected with 0.5-percent diethylstilbestrol pellets.

The most common tumors other than mammary adenocarcinomas were hepatomas. Data on the occurrence of hepatomas are summarized in table 2. The incidence of hepatomas is lower than recorded for normal males of the C3H strain. No hepatomas were encountered in animals less than 19 months old that received estradiol or diethylstilbestrol pellets, whereas Andervont (*6*) in 1941 reported an incidence of 25.8 percent in males from 11 to 19 months of age. Female mice of the same age had an incidence of hepatomas of 5.7 percent.

TABLE 2.—*Hepatomas in male C3H mice implanted with estrogen-cholesterol pellets*

[Numerator = mice with hepatomas. Denominator = mice dying or killed]

Concentration of estrogen in pellet (percent)	Estrogen in pellet	Age of mice in months				Total mice	Total hepatomas
		13–15	16–18	19–21	22–24+		
	Mg.					*Number*	*Number*
DIETHYLSTILBESTROL							
0.5	0.01	0/5	0/3	2/16	1/12	36	3
1.0	.02	0/2	0/7	1/14	0/10	33	1
2.0	.1	0/3	0/2	1/7	1/9	21	2
5.0	.04	0/3	0/7	1/2	3/11	23	4
10.0	.2	0/7	0/3	0/2	0/2	14	0
20.0	.4	0/3	0/1	0/3	-------	7	0
Total	-------	0/46		10/88			
ESTRADIOL							
1.0	.02	0/8	0/10	5/12	0/5	35	5
2.0	.04	0/5	0/3	2/8	0/5	21	2
5.0	.1	0/7	0/1	0/2	1/2	12	1
10.0	.2	0/1	0/1	0/1	0/1	4	0
20.0	.4	-------	-------	-------	0/1	1	0
Total	-------	0/36		8/37			
TRIPHENYLETHYLENE							
10	.2	0/3	0/3	0/2	4/15	23	4
25	.5	1/2	1/4	0/3	5/18	27	7
50	1.0	0/4	0/4	0/3	1/14	25	1
Total	-------	2/20		10/55			

Thus, the later appearance and lower incidence of hepatomas in this experiment may be an effect of estrogenation. It may be of significance that the groups implanted with triphenylethylene, the weakest estrogen, were the only ones in which hepatomas occurred in mice under 19 months of age. There is no evident correlation between the dose of estrogen and the reduced incidence of hepatomas, however, and the incidence at older ages was the same in the estradiol and the triphenylethylene groups. Most important, no untreated normal controls were maintained for the essential comparison, and conclusions regarding the effect of estrogens on the occurrence of hepatomas in male mice must await further investigation.

Incidental tumors encountered in the mice of this experiment included occasional pulmonary tumors, the subcutaneous sarcoma referred to previously, an epidermoid carcinoma of the skin of the right foreleg in a mouse, 23.5 months old, in the group receiving 1.0 percent diethylstilbestrol pellets, and two lymphomas that arose in the 1.0- and 2.0-percent estradiol-pellet groups when the animals were 15.3 and 12.0 months old, respectively. The only tumors requiring comment are the lymphomas.

Investigations with estrogens at the National Cancer Institute, using C3H mice of the Andervont subline, have elicited no significant increase in the incidence of lymphomas. In 3 previous experiments (*4, 7*), no lymphomas arose in 22 and in 45 mice treated with estrogens, and 2 lymphomas were found in 1 group of 30 mice implanted with estrone crystals. The combined incidence, therefore, was 2.0 percent (2 of 97 mice). The present incidence is 0.6 percent (2 of 320 mice), if only mice surviving for 6 months or longer in the diethylstilbestrol and the estradiol groups are included. These data are in contrast with the results obtained at Yale (*8*) with a different line of C3H mice; Gardner reports an incidence of lymphomas of 15.4 percent (22 of 140 mice) in C3H mice, which had received variable amounts of estrogen, in comparison with the occurrence of 1 lymphoma in 203 normal animals.

SUMMARY

Male mice of strain C3H (Andervont subline) were implanted with single pellets of cholesterol weighing 2 mg. and containing from 0.5 to 50 percent of diethylstilbestrol, estradiol, or triphenylethylene; 149 mammary tumors were induced in 461 mice at average latent periods ranging from 5.9 months for the higher doses of estradiol to 16.8 months for the lower doses of diethylstilbestrol.

The average absorption of the pellets was 10 percent. The dose producing mammary tumors in 50 percent of the animals was calculated to be between 4 to 10 micrograms of estradiol and between 20 and 40 micrograms for diethylstilbestrol.

Five percent of the animals developed mammary tumors with an absorbed dose of 2 micrograms of estradiol or diethylstilbestrol. No tumors were elicited with triphenylethylene.

The carcinogenic dose of estrogens for male mice of the C3H strain is well within the range of concentrations elaborated physiologically by normal female mice.

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MAMMARY TUMORS IN MICE FOLLOWING TRANSPLANTATION OF MAMMARY TISSUE[1]

By MICHAEL B. SHIMKIN, *surgeon*, ROSE S. WYMAN, *laboratory technician*, and H. B. ANDERVONT, *principal biologist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The experiment reported herein was made to ascertain whether mammary tumors would arise from epithelial tissue of mammary glands transplanted into other mice. It was hoped that this would allow observations in vivo of the development of such tumors, as well as furnish a technique for the study of various interrelationships between hormones and the development of mammary tumors, and the effects of age of the tissue and of the host in mammary carcinogenesis.

Although under the conditions of the experimental method the purpose was not achieved, the results elicited a number of interesting implications.

EXPERIMENTAL PROCEDURE

Male mice of strain C3H (Andervont subline), freed of the milk agent, were used. The animals were second-to-sixth-generation descendants of a C3H litter that was removed from the uterus and foster nursed by a strain C57 black female. The mice were used when they were 4 to 8 weeks old. They were kept in wooden boxes with wood shavings as bedding, five to eight animals to a box, and were maintained on Purina laboratory chow pellets and had an unlimited supply of water.

The mammary tissue for transplantation was obtained from virgin females of the C3H strain 1.3 to 7.5 months of age. The mice, which were normal females harboring the milk agent, were killed by cervical dislocation, and the subcutaneous tissue containing the mammary glands was removed. The epithelial tissue of the mammary glands was freed as much as possible from the connective tissue, under a dissecting microscope.

The hair of the abdomen of the male mice was clipped and the area washed with alcohol. A single mammary duct and its terminal branches obtained from a female were implanted subcutaneously by means of a small forceps, through a 3-mm. horizontal midline incision. The site in the midline between the thoracic and inguinal regions was selected because it is one of the few areas of the body of the mouse in which mammary tissue is not present. At the same time, a single 2-mg. pellet of cholesterol containing 20 percent diethylstilbestrol was introduced into the right axilla in order to furnish adequate estrogenic stimulation for the development of the mammary tissue.

The mice were examined by palpation at weekly intervals. The latent periods were determined by the time at which definite subcutaneous masses were felt. Tumors and other tissues that in the gross appeared abnormal were examined under the microscope after appropriate fixation, sectioning, and staining. All diagnoses were accepted only on the basis of histologic examination.

RESULTS

The results are presented in table 1. Of 67 mice that survived 8 months or longer, 13 developed mammary tumors, an incidence of 19.4 percent. Only 1 of these tumors, however, arose exactly at the site of the transplanted tissue. This tumor was elicited in a mouse implanted with a mammary duct from a 74-day-old female and was detected 24.3 months following the implantation of the mammary tissue and the estrogen pellet. The tumor was transplanted successfully into other C3H mice and was discontinued after 4 transplantation generations.

The remaining 12 mammary tumors appeared in normal sites of mammary tissue and could not be considered as arising from the transplanted tissue. The distribution of the tumors was as follows: right axilla, 4; left axilla, 5; right inguinal

TABLE 1.—*Mammary tumors in male C3H mice, free of milk influence, implanted with mammary duct of normal C3H female mice and a 20-percent diethylstilbestrol cholesterol pellet*

[Numerator = Mice with mammary tumors. Denominator = Mice dying without mammary tumor]

Age of female donor of breast tissue (in months)	Appearance of mammary tumors in months after breast transplant																	Total mice	Tumors		Average latent time
	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24+		*Number*	*Percent*	*Months*
1.3	1/0	----	0/1	----	0/1	----	----	----	----	----	----	1/1	0/1	0/2	0/1	----	0/2	11	2	18.2	13.7
2.5	0/3	1/0	----	1/0	----	1/3	----	----	1/0	1/1	----	----	0/1	0/1	0/2	0/1	1/8	26	6	23.1	15.6
3.3	----	----	----	0/2	----	0/1	----	----	1/0	0/2	----	----	0/1	0/1	1/1	----	0/1	11	2	18.2	19.0
4.0	----	----	----	----	2/0	0/1	0/1	----	0/1	1/0	0/2	----	----	----	----	----	0/4	12	3	25.0	13.7
7.5	0/1	0/1	----	----	----	----	----	----	0/2	----	0/2	----	----	----	----	----	0/1	7	0	----	----
Total or average																		67	13	19.4	15.3

[1] Received for publication September 16, 1946.

region, 2; and right lower flank, 1. Sections through the transplant areas showed survival of the duct epithelium. Interpretation of the failure in this experiment to elicit mammary tumors at the site of transplanted mammary tissue in all but 1 instance must be limited to the specific experimental procedures that were employed.

The procedure increased the incidence of mammary tumors that could have been expected in C3H male mice lacking the milk agent. Normal strain C3H females develop mammary tumors in an incidence of over 90 percent at an average age of 8.6 to 10.4 months (*1*). Normal C3H males implanted with 20-percent diethylstilbestrol-in-cholesterol pellets develop mammary tumors in an incidence of 75 percent at an average age of 8.4 months (*2*). Descendants of the C3H mice that were removed from the uterus and foster nursed by the C57 black female are now in the F_{12} generation. The breeding females of the first 7 generations, including litter mates of the males used in this experiment, total 200 animals, and of these 7, or 3.5 percent, developed mammary tumors at an average age of 22 months. Male mice injected with estrogen pellets, therefore, would not be expected to develop mammary tumors in over 3.5 percent of the animals.

It is concluded that the transplantation of mammary ducts from female mice harboring the milk agent into estrogenized males lacking the milk agent infects such mice with a sufficient amount of the agent to raise the incidence of mammary tumors to approximately 20 percent. The milk agent apparently is not fixed in the mammary epithelium but can be transmitted to other mammary tissue and elsewhere in the body under the conditions of the experiment. The milk agent is widely distributed in the body of the mouse, yet it is thus far known to be involved only in the genesis of mammary tumors (*3*, *4*).

Mammary tissue obtained from mice of ages varying from 1.3 to 4.0 months produced closely similar results. The lack of tumors in seven mice implanted with mammary tissue from a 7.5-month-old female cannot be commented upon until the observation is repeated with larger numbers of animals.

Three other subcutaneous tumors arose in the mice used in this study. None of these tumors was in the region of the transplant, and all were evidently spontaneous in origin. A spindle-cell sarcoma arose on the posterior cervical area of a 22-month-old mouse. An osteogenic sarcoma involving the right thoracic wall and foreleg was found in a mouse 24 months old. In the third mouse, the multiple subcutaneous masses were found to be lymph nodes associated with the involvement of the spleen and other internal organs by lymphoma.

A subcutaneous spindle-cell sarcoma has occurred in 1 of 180 mice of a previous experiment (*2*) surviving 19 months or longer, and 3 subcutaneous sarcomas have appeared at an average age of 20.5 months among the 200 female descendants of the C3H mice that were removed from the uterus and foster nursed by the C57 black female. Occurrence of a subcutaneous osteogenic sarcoma in a C3H mouse also has been described (*5*).

SUMMARY

Strain C3H male mice, free of the milk agent, were implanted in the midline of the abdomen with single ducts of breasts from normal C3H females 1.5 to 7.5 months old, and in the right axilla with 20-percent diethylstilbestrol-in-cholesterol pellets. Of 67 mice, 13 developed mammary tumors, but only 1 of the tumors appeared at the site of the mammary-tissue transplant.

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PATHS OF GENE ACTION IN MAMMARY-TUMOR DEVELOPMENT IN MICE[1,2]

By WALTER E. HESTON, *principal geneticist, National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

The etiologic factors of mammary tumors of the mouse have been investigated more extensively than those of any other tumor. The hormonal influence has attracted the endocrinologist, and the milk agent the virologist and biophysicist, while even the nutritionist has found this tumor responsive. But throughout the whole study, the work of the geneticist has been basic.

In early investigations it was found that inbreeding with selection resulted in certain strains in which the incidence of mammary tumor was high as contrasted with other strains in which the incidence was extremely low, thus emphasizing the importance of the genetic constitution. Reciprocal crosses between these high- and low-tumor strains, however, revealed the presence of the maternally inherited milk agent; and subsequent investigations have shown that normally the chances are slight for mammary-tumor development in an individual mouse that has not received the milk agent. Nevertheless, there is now ample evidence that the genetic factors are of greater basic importance in controlling tumor incidences and maintaining a high-tumor strain.

Now that genetic differences have been demonstrated and confirmed the present problem is that of determining the physiologic mechanisms through which these variations in genic complex can and do effect mammary-tumor development. From recent work on gene chemistry,[3] it is reasonable to expect that these genic differences can be manifested as enzymatic differences affecting specific metabolic reactions leading to the development of the tumor. It is hoped that some day these chemical reactions will be revealed and that the specific genes will be identified with their associated enzymatic actions. Although one should maintain a full appreciation of the ground yet to be covered before this goal can be reached, one can now profit from a survey of the problem, for it can be seen that at this point we have accumulated evidence that indicates the general fields in which certain of the genic differences are manifested. The identification of these fields opens leads toward the discovery of the primary genic actions.

The relationship of the genetic factors to the eventually developed mammary tumor as it appears today is presented in figure 1. By the union of the ovum and sperm from which the mouse originates, there is established a certain complex of genetic factors. This complex is passed from cell generation to cell generation throughout the life of the organism, and at various times certain of these genes enter into the physiology of the organism in such manner as to influence the chances that the mouse will develop a mammary tumor.

Soon after birth the mouse begins to nurse upon its mother and in so doing receives the milk agent if the mother carries the agent. For the development of most mammary tumors in mice, this agent appears to be essential. However, the probabilities that the introduction of the agent will result in a mammary tumor is dependent in part upon the type of genes with which the agent finds itself associated. Experiments in which maternal influence was kept constant have demonstrated that genetic differences alone as affected by the genetic differences of the fathers can markedly affect the chances of the offspring's developing a tumor. Furthermore, there is evidence that the genes influence the degree to which the mouse will be able to transmit the agent to future generations. Thus, one of the paths through which genic differences influence the probability for the appearance of the tumor is through the physiology involving the milk agent.

As the mouse matures, hormonal stimulation exerts its influence. If proper stimulation is lacking, as in the males, the mammary tree fails to develop, and hence tumors do not appear; but in addition to this, there is generally a variation between the tumor incidence of the breeders as compared with that of the virgins within the various high-tumor strains. Here again there is evidence that the effect manifest through hormonal physiology can be altered by altering the genetic constitution alone. Thus, a second path of genic action is demonstrated in the physiology of the hormonal stimulation.

The processes leading to the appearance of the mammary tumor continue. The hormones and the milk agent exert their stimulations on the mammary tissue, but the response of the mammary tissue to these stimuli appears to be influenced by the so-called susceptibility of the mammary tissues. This susceptibility we are inclined to consider as an expression of the genes within the mammary-tissue cells. The paths of action of these genes are probably limited to within these cells.

Thus the picture unfolds. Whereas in the past there was an inclination to place the milk agent, the hormones, and the genetic constitution more

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[2] Paper presented at the American Association for the Advancement of Science, Gibson Island Conference on Cancer, August 12–16, 1946.
[3] For a review of the subject, see Beadle (*1*).

FIGURE 1.—Gene-mammary tumor relationship.

or less parallel, from more recent findings it now appears that we should picture the genes in a more basic position, with their actions becoming manifest through at least three gene-action paths, the first involving the milk agent, the second the hormonal stimulation, and the third the susceptibility of the mammary tissue. How many more such paths there will be can be answered only through further experimentation. There is no reason to believe that the picture is now stabilized; and let us hope that it is not, for as the development processes are further divided, one approaches the identity of the specific metabolic reactions with which the specific genes are associated.

The data on which this present concept has been formulated are briefly reviewed in the following sections.

GENETIC DIFFERENCES IN SUSCEPTIBILITY OF THE MAMMARY TISSUE

For the past decade one of the three primary factors considered as influencing mammary-tumor production has been the genetic susceptibility (*2*). While this genetic susceptibility has not been defined in detail, the implication has been in part that it constituted a genetic control over the response of the mammary-tissue cell to the carcinogenic stimuli, the more direct one being the milk agent and the other the hormonal stimulation.

The concept of this avenue through which genes are related to the tumor is still the most indefinite of the three described herein, but as is shown in the two subsequent sections, there is a suggestion of a genetic variation in the response to the hormonal stimulation. Furthermore, there is evidence that with the suitable hormonal influence present, a mouse may possess the milk agent, as indicated by the production of tumors in test females, but yet does not develop a tumor because her mammary tissue is not genetically susceptible to the action of the milk agent.

Whatever the chemical reactions may be through which the genic differences appear, the logical place in which to look for them is in the physiology of the mammary-tissue cells. Genic differences in response to the hormonal stimulation could be expected to appear in the chemistry of these cells at the time when the mammary tree is forming,

whereas genic differences in response to the milk agent are probably manifest through their chemistry at a time when we presume that the milk agent is acting upon them. Thinking in terms of general biologic response, one might be inclined to picture this action of the agent as occurring at or near the time of the malignant transformation; but if one attempts to think in terms of a specific chemical reaction in which the milk agent is effective, he realizes that there is no definite proof that the action of the milk agent does not come earlier. The primary genic action involved in the response to either the hormonal or milk-agent stimulation, however, must take place in the physiology within the mammary-tissue cell.

GENETIC DIFFERENCES IN HORMONAL STIMULATION

That genetic differences can influence mammary-tumor development through the hormonal mechanism has been demonstrated by crosses between strains A and C3H, by Bittner et al. (*3*, *4*), and Heston and Andervont (*5*). Both of these strains are high-mammary-tumor strains possessing the milk agent in apparently equal concentrations; but whereas the incidence for both breeding and virgin C3H females is high, only the breeding strain A females have a high incidence while that of the virgins is less than 5 percent. This suggests a difference in amount of hormonal stimulation especially since estrogens administered to the virgin strain A females raise the incidence, and furthermore, since Jones (*6*) has demonstrated for strain A a quantitative relationship between tumor incidence and number of pregnancies.

That this difference was fundamentally genetic was shown by reciprocal crosses between the two strains (table 1). Both types of F_1 virgin females had a high tumor incidence approximating that of the C3H. The results of the F_1 virgin females derived from strain A mothers mated to C3H fathers showed that the constituents of the sperm alone from the C3H fathers were able to raise the incidence from the low percentage of the strain A virgin females to a high percentage comparable with that of the strain C3H. Since it is generally accepted that an influence introduced by the sperm is a genetic influence, it can be assumed that this effect was owing to the genes transmitted by the C3H father.

TABLE 1.—*Evidence of genetic difference in mammary-tumor incidence manifest through hormonal stimulation* [1]

Virgin females	Number	Cancer incidence	Average cancer age	Average noncancer age
		Percent	*Months*	*Months*
Strain A (*5*)	22	0	.....	16.4
A, foster-nursed by C3H (*5*)	32	16	10.6	16.9
$AC3HF_1$ [2] (*5*)	45	91	15.8	21.3
Strain C3H (*5*)	29	93	10.9	14.0
C3H, foster-nursed by A (*5*)	35	94	11.5	17.5
$C3HAF_1$ [3] (*5*)	36	83	13.8	14.3
Strain A (*4*)	102	3.9	15.0	19.3
Strain C3H [4] (*4*)	92	63.0	13.3	15.9
$AC3HF_1$ [2] (*4*)	93	92.5	15.2	18.9
$C3HAF_1$ [3] (*4*)	56	73.2	19.1	22.5
$AC3HF_2$ (*4*)	98	70.4	16.2	21.8
$C3HAF_2$ (*4*)	98	38.8	17.2	22.0

[1] Data from Heston and Andervont (*5*) and Bittner and Huseby (*4*). In the first column, reference is indicated by italic number in parentheses.
[2] Parents, A ♀ × C3H ♂.
[3] Parents, C3H ♀ × A ♂.
[4] C3H or Z.

In the data of Bittner and Huseby there was a difference between the reciprocal F_1 hybrids, those with strain A mothers having a higher tumor incidence than those with strain C3H mothers, and this difference again appearing in the F_2 generation. Bittner and Huseby suggested that although a difference in milk agent was not indicated by the results of Heston and Andervont, such a difference might account for their results. The two sublines of strain C3H differ in tumor incidence.

These observations have led to the problem of locating where the gene action affecting these differences occurs. Are these genic differences affecting hormonal metabolism resulting in a more optimum output in the strain C3H, or is the hormonal stimulation in strain A virgins identical with that that of C3H virgins and do the A females fail to get tumors because their genetic constitution renders their mammary-tissue cells more resistant, thus requiring more stimulation to give the same response as that of the C3H? As yet these questions are not answered fully. The observations cited previously suggest that the difference is in the amount of hormone produced, but they do not rule out the possibility of variation in sensitivity. It is highly probable that genic differences are affecting both stimulation and sensitivity.

The amount of stimulation could be most accurately measured by assay of estrogen production of the females of the two parent strains and the reciprocal hybrids. For an animal the size of the mouse, however, such assays would require special techniques, but the results would undoubtedly justify their perfection. Some information has been obtained through the more simple approach of observations of estrus cycles. Deringer et al. (*7*) observed the onset of estrus and followed the cycles in strain A and strain C3H virgin females and the virgin females from reciprocal crosses between the two strains, through 150 days of age. After onset of estrus, no difference in the characteristics of the cycles of the four groups could be found. There was, however, a significant variation in the ages at which the vagina opened and hence the cycle began. In the three groups with

high tumor incidence, it was found that the opening of the vagina occurred on the average from 9 to 14 days earlier than it did in the strain A virgins with low tumor incidence.

Such findings indicate a difference in hormonal stimulation, but the difference was not great. Thus, it appeared that a major part of the genetic difference might yet be manifest as difference in susceptibility to the stimulation unless it could be shown that this was a critical age at which slight differences in hormonal stimulation had a great effect in determining whether or not the female would later develop a tumor.

GENETIC DIFFERENCES IN ABILITY TO PROPAGATE AND TRANSMIT THE MILK AGENT

The third avenue through which genic differences can alter the probability that a mouse will develop a mammary tumor is through the relationship of the gene to the milk agent. This was shown in a recent detailed study (*8*) in which backcross females of equal maternal influences but of unlike genetic constitutions were compared as to their tumor development and their ability to transmit the milk agent (table 2).

TABLE 2.—*Evidence of genetic difference in ability to propagate and transmit the milk agent* [1]

Type of group	Nursed by—	Number	Tumorous		Nontumorous	
			Percent	Age	Percent	Age
				Mos.		*Mos.*
C3H (Breeders)	C3H (Mother)	14	93	9.6	7	11.0
C3H × B)F$_1$ (Breeders).	do	52	96	12.9	4	24.5
(C3H × B)F$_1$ (Virgins)	do	30	90	14.2	10	23.7
(C3HB × C3H)BC (Breeders).	(C3H × B)F$_1$ (Mother)	88	93	11.6	7	19.3
(C 3 H B × B) B C (Breeders).	do	98	55	16.6	45	21.6
(B × C3H)F$_1$ (Virgins)	(C3HB × C3H)BC (Foster mother)	375	90	14.9	10	21.3
Do	(C3HB × B)BC (Foster mother)	355	68	15.0	32	24.7

[1] Data from Heston et al. (*8*).

The two groups of backcross females were developed by mating high-tumor strain C3H females to low-tumor strain C57 black males, and in turn backcrossing the resulting F$_1$ females with C3H males to obtain the susceptible-strain backcross females, and with C57 black males to produce the resistant-strain backcross females. When these two groups were compared as to tumor incidence, it was found that although both groups had received comparable milk-agent and other possible maternal influences, the group with the more chromatin from the susceptible strain C3H had a tumor incidence approximately 40 percent higher than that of the group with more resistant-strain chromatin. This was a marked difference that could be attributed solely to a genetic difference.

It was found furthermore that the backcross females with the higher percentage of susceptible-strain chromatin were able to transmit the agent more effectively as measured by the tumor development in uniform, susceptible, test females, the F$_1$ hybrids from C57 black females mated to C3H males. Thus, although the milk agent that both groups had received was comparable, it had survived or propagated better in the mice with the more susceptible-strain chromatin. Moreover, there was within each backcross group variation in degree to which the females transmitted the agent, indicating the result of segregation of multiple genes (table 3).

TABLE 3.—*Variation in the milk agent transmitted by the two types of backcross females* [1]

Type of foster mother	Number with given percent of tumorous fostered females										
	0	20	25	33⅓	40	50	60	66⅔	75	80	100
(C3HB × C3H)BC:											
Tumorous							7	2	1	7	53
Nontumorous	1	2								2	1
Total	1	2					7	2	1	9	54
(C3HB × B)BC:											
Tumorous						1	1	2	2	8	25
Nontumorous	8	1		2	5	2	3		4	4	6
Total	8	1		2	5	3	4	2	6	12	31

[1] Data from Heston et al (*8*).

All the backcross females of both groups which later developed tumors transmitted the agent effectively, as indicated by the high percentage of their test females that developed tumors. Of the backcross females that failed to develop tumors some were also able to transmit the agent, for tumors appeared in their test females; but on the average the percentage was lower for these test females than for those nursed by the backcross females that did develop tumors. In this observation, there was a suggestion that two sets of genes were involved, one influencing the propagation of the agent and the other the response of the mammary tissue to the agent. Accordingly all females possessing both sets of genes would develop a tumor if given the agent and would also be able to transmit the agent. Those lacking the genes for propagating the agent would neither develop a tumor nor transmit the agent, but those lacking the genes for the response of the mammary tissue to the agent but possessing the genes for propagating the agent would be able to transmit the agent although they remained nontumorous. If such

is the case, there must be one or more common factors since the nontumorous females that did transmit the agent did so less effectively than the females that later developed tumors. It is conceivable, however, that this indication of two sets of genes may be owing to variation in response brought about by variation in unknown nongenetic factors.

In the genic influence on the transmission of the milk agent was seen an example of a gene-action path extending from one generation to the next, the type of condition which Russell (*9*) recently suggested would be possible. In the analysis of the tumor development in the test female, it was seen that the variation could not be attributed to genetic differences of the test females since they were genetically alike, but had to be the result of a variation in the milk agent that they received. This variation in milk agent was in turn brought about by the genetic variation of their backcross foster mothers. Here genic action in one generation became manifest in the following generation (in this case the fostered generation) through a medium other than the sex cells. The genetic constitution of the foster mothers acting through what the present author (*10*) included in biologic environment influenced the subsequent development of the character in the fostered females.

The observations recorded here are not alone in demonstrating the importance of the genetic constitution in the propagation of the milk agent. When Murray and Little (*11*) built up a resistant-strain genetic background for the milk agent by starting with susceptible-strain dba females mated to resistant-strain C57 black males and following with eight generations of backcrossing to the resistant-strain males, they found that the eight-generation females with a proportion of C57 black chromatin that approached 100 percent no longer had the milk agent. In the work described in the preceding paragraphs, this condition apparently was attained in some of the first-generation backcross females. Andervont (*12*) found that although resistant-strain C57 black females that had nursed upon strain C3H females could transmit the agent to susceptible young, their later generation C57 black offspring could not, a fact that indicated that continued propagation of the agent was not compatible with the genotype of this substrain of C57 black.

Somewhat in contradiction to the general picture, however, are the observations of Fekete and Little (*13*), who reported a relatively high incidence of mammary tumors in three generations of females descended from C57 black females that had developed in strain dba uteri from transferred ova.

Andervont (*12, 14*) found that low-tumor-strain C was changed to a high-tumor strain by the introduction of the milk agent from strain C3H. This change could be expected if strain C was genetically susceptible to the agent and had remained a low-tumor strain merely because the agent had not been introduced.

Thus there is evidence of genetic variation in the survival of the milk agent, which is so great that from one generation to the next one can apparently eliminate the agent in certain of the females merely by altering the genetic constitution. Just what the genic action is in relation to the propagation or elimination of the agent we do not know, nor can the action be localized to a particular tissue or organ. The agent has been demonstrated in various tissues throughout the body, and there is no reason to suspect that it is not propagated in these tissues as well as in the mammary tissue.

As pointed out recently, this gene-milk agent relationship is not unique. In virus studies the genetic constitution of the host and even of the vector is often found to be important. Gowen (*15*) stated that in their laboratory disease is considered to be due to the interaction of four factors, the genetic constitution of the host, the genetic constitution of the pathogen, the dose of the pathogen, and a fourth group combined under the head of environmental effects. In general, this concept is sound. However, in view of apparent inability of the milk agent and viruses in general to metabolize or reproduce outside the living host cell, one questions whether they should be considered as having a distinct genetic constitution or whether their variation may be an expression of genetic variation of the hosts.

Considerable emphasis has recently been given to discussions of comparisons between such agents and so-called plastogenes and plasmagenes. There is a high similarity between the relationship of the genes to the milk agent and that of Sonneborn's genetic factor to the cytoplasmic factor in *Paramecium* (*16*). Murphy (*17*) emphasized earlier the intimate relation between the host cell and the Rous chicken-tumor agent and suggested that the tumor agents may be of endogenous origin. Comparisons of the gene-milk agent relationship to these and other similar situations are of value in that they emphasize the necessity of portraying the milk agent as a part of the cell and as an agent which may have arisen by a change in some normal cellular component. Furthermore, while crystallization studies will be extremely valuable, emphasis must be placed on the milk agent, not as an entity in itself but as one unit among the physiologically co-ordinated nuclear and cytoplasmic units that comprise the cell.

POSSIBLE RELATION TO HUMAN BREAST-CANCER PROBLEM

Since the ultimate purpose of cancer research is to contribute to the understanding of cancer development in man, one is justified in asking what have been the contributions of these studies toward the understanding of the breast-cancer problem in the human being. At the outset, one must bear in mind that mice are genetically mice and men are genetically men, and obviously direct application cannot be made from mice to men. However, investigations with the mouse have been of value in conditioning our view with regard to the type of of problem we can expect in breast cancer in man and have suggested certain projects worth undertaking.

Any hope of finding segregation ratios of breast cancer in man with a view of making eugenic applications now appears futile. For any character preceded by such a complex physiology in which so many genic differences are probably expressed, one would not be able to predict ratios. If, however, in man this physiology leading to breast-cancer formation is broken down, as it is being broken down in the mouse, it may be that certain paths can be found through which simple genic differences are expressed as conditions favorable to cancer development as opposed to those unfavorable. A knowledge of such conditions would be of extreme value, for with the aid of family histories such conditions might be revealed early in life, and possibilities for preventive treatment might be opened.

On discovering that the maternal influence in the mouse was transmitted through the milk, Bittner (*18*) conservatively stated that a possible application would be the suggestion that mothers of breast-cancer families should not nurse their babies. Others (*19*, *20*) have gone so far as to stress such a recommendation. Bittner's conservatism appears justified, if one considers that at present a milk agent has not been demonstrated for breast cancer in man and that furthermore there is little reason to suspect that if an extrachromosomal agent is demonstrated, it would be any more likely to be transmitted through the milk than through any one of a number of other possible channels, including the uterine wall or the cytoplasm of the egg. Nevertheless, since this agent was discovered in the mouse, the search for a milk agent or any other extrachromosomal factor should have prominent consideration in any human-breast-cancer study.

Undoubtedly the greatest contribution to the problem of breast cancer in the human being made by the laboratory investigations has been the conditioning of our view of the probable nature of the human cancer problem. While control of breast cancer in man may come through some isolated discovery, there is greater probability that it will come through a more thorough understanding of the physiology leading to the development of the cancer. As in the mouse, this physiology must be extremely complex. Hormonal factors undoubtedly play a role. Extrachromosomal factors passed from parent to offspring or at least from cell generation to cell generation must have a part. They may not be of a classical virus nature, but there surely will be altered cytoplasmic components to be taken into consideration. Just as in the mouse, it will probably be found that these factors enter into a physiology in which genic action is of great importance, for throughout the ramifications of this physiology genic differences will surely be found. Thus, as has been experienced in the mouse, the discovery of nongenetic factors well may come through the study of the genetic differences. It is therefore reasonable to expect that one of the most fruitful attacks on the problem of breast cancer in man will be through the genetic approach.

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GENETICS OF OCULAR-TUMOR DEVELOPMENT IN FISHES (PRELIMINARY REPORT)[1] [2]

By MYRON GORDON, *research associate in genetics, New York Aquarium, New York Zoological Society*

INTRODUCTION

In a recent paper, Levine and Gordon (*1*) described the histologic features of ocular tumors in a number of platyfishes, swordtails, and their hybrids. Some of them were found in broods of which all but one member were normal. Two of the diseased fish, called hybrids A and B, were genetically related, having the same grandparents. Subsequently more fish with exophthalmic tumors were found in related stocks. This finding suggested a common genetic origin for most of the fishes that developed ocular tumors.

The present paper reports the available genetic data on this subject and points out that the genetic influence might be the determining factor in the development of these ocular tumors.

MATERIALS

The broods of which hybrids A and B were members showed a higher incidence of ocular tumors than was expected. These hybrids originated from a mating between a female albino (*ii*) swordtail, *Xiphophorus hellerii*, culture No. 124-2, and a male spotted-belly (*Sb*) and spotted-dorsal (*Sd*) platyfish, *Platypoecilus maculatus*, culture No. 37 [2]-11. These parents are represented in figure 1 on the top line of the diagram, and by their photograph in figure 2.

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[2] Aided in part by grants from the Anna Fuller Fund and the American Cancer Society on recommendation of the Committee on Growth, of the National Research Council. The work was aided by the facilities of the Departments of Birds and Animal Behavior, of the American Museum of Natural History.

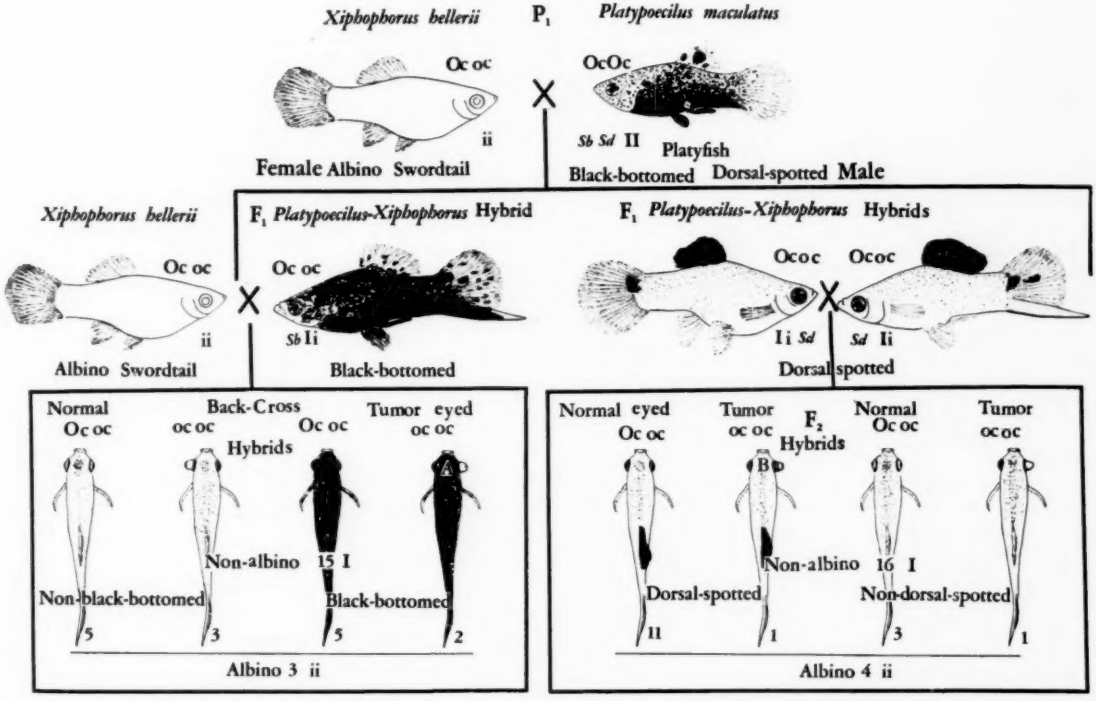

FIGURE 1.—Genetic history of two platyfish-swordtail broods which contained an unusually high number of fish showing ocular tumors with exophthalmia. Genetic symbols: *II* and *Ii* are equivalent to ++ and +*i* in the text, and all denote non-albinism; + *OcOc* and *Ococ* are equivalent to ++ and + *oo* in the text and denote normal-eyed, while *ococ* denotes ocular tumor; *Sb* denotes spotted-belly or black-bottom; *Sd* denotes spotted dorsal fin.

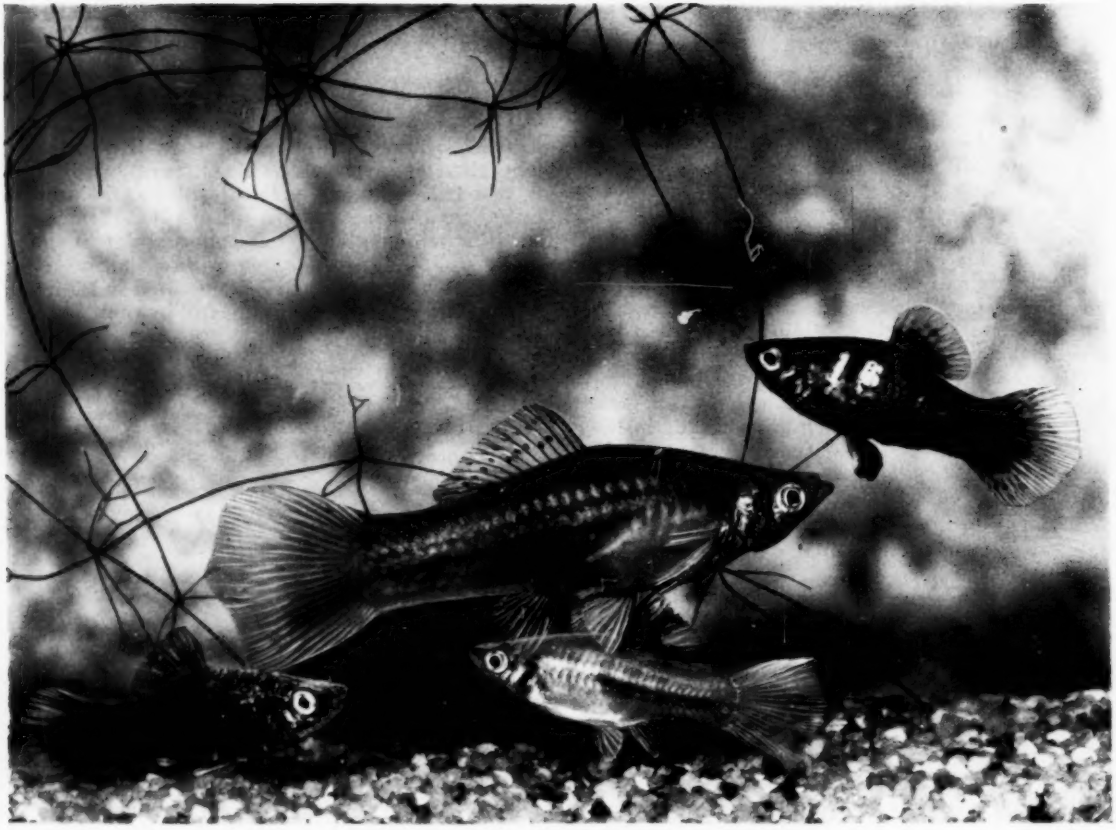

FIGURE 2.—Normal-eyed parents and grandparents of broods in which significant numbers developed ocular tumors with exophthalmia. The grandparents are the upper two, a female albino swordtail (left) and a black-bottomed, dorsal-spotted male platyfish (right). They produced two groups of hybrids represented by the black-bottomed hybrid (bottom, left) and the dorsal-spotted hybrid (bottom, right). The second-generation offspring are shown in figure 1. (Photograph by Sam Dunton, staff photographer of the New York Zoological Society).

The platyfish-swordtail F_1 hybrids were of two phenotypes: about half of them developed melanomas of the mid-ventral region (illustrated in figure 1 by the second fish from the left on the second line) while the other half developed melanosis of the dorsal fins (shown by the third and fourth fish from the left on the second line). A preliminary report on the influence of the *Sb* and *Sd* genes on the development of regional melanotic tumors was made by Gordon (*2*). None of the F_1 hybrids developed visible ocular abnormalities although they attained an age of 18 months or over, an age that represents post-middle life for these fish. The photograph of the hybrids (fig. 2) was taken when they were 8 months old and when the melanotic tumors on the belly region of one and on the dorsal fin of the other were beginning to show.

GENETIC ANALYSIS

From subsequent events, particularly the development of ocular tumors in later generations, the original mating of the albino swordtail female and the spotted platyfish male may be written as follows:

$$\underset{\text{♀♀}}{ii + oc++} \times \underset{\text{♂♂}}{++\ ++\ SbSd.}$$

where *ii* (homozygous recessive) is albinism and $+i$ or $++$ is normal color, gray; $+oc$ or $++$ is normal-eyed, the former being heterozygous for ocular tumor; *Sb* is spotted-belly or black-bottom;

Sd is spotted-dorsal; *Sb* and *Sd* are treated as alleles.

The genotypes of the F_1 hybrid fish expected may be listed as follows:

(1) $+i$ $++$ $Sb+$, normal color, normal eyes, spotted-belly;
(2) $+i$ $+oc^*$ $Sb+$, normal color, normal eyes*, spotted-belly;
(3) $+i$ $++$ $Sd+$, normal color, normal eyes, spotted-dorsal;
(4) $+i$ $+oc^*$ $Sd+$, normal color, normal eyes*, spotted-dorsal.

The two groups starred (*), while they are normal-eyed, carry the assumed recessive gene *oc* for ocular tumor in a heterozygous condition.

GENETIC HISTORY OF HYBRIDS

Hybrid A

Hybrid A was one of the fish with an ocular tumor described by Levine and Gordon (*1*). The tumor produced blindness in the right eye of the fish, which caused it to swim in circles. This is illustrated in figure 3, *a-f*. The fish was one of a brood derived from the mating of an albino swordtail female (fig. 1, to the extreme left on line 2) with a spotted-belly platyfish-swordtail male hybrid (fig. 1, second from the left). The parentage of hybrid A may be expressed as follows:

$$\overset{♀♀}{ii\ +oc\ ++} \times \overset{♂♂}{+i\ +oc\ Sb+}$$

Fifteen of their young were reared to the age of 12 months or over; 5 of the 15 developed ocular tumors, a number far greater than one might expect on the basis of experience in rearing thousands of these platyfish-swordtail hybrids and other xiphophorins. The number and the frequencies of the various phenotypes obtained are given in table 1. The 4 phenotypes are shown in figure 1 under the heading "back-cross." In addition to the 15 adult hybrids mentioned, there were 3 albino fish in the same brood, but they died within the week of birth; the lack of viability of albinos in some broods was noted previously by Gordon (*3*). These albinos might have been in any of the 4 categories listed (table 1), but phenotypically they were similar. These are noted in figure 1 but not illustrated.

When the "goodness-of-fit" was derived on the basis of all the observed members of the brood, including the few inviable albinos, the value of χ^2 was 9.37, and of P 0.05, just lacking significance. But when the albinos were not counted, that is, when based on 15 mature fish, the value of χ^2 was 0.823, and of P 0.85, and hence significant.

TABLE 1.—*Number and frequencies of observed and expected phenotypes based upon the assumed constitution of the parents*[1] *of hybrid A*

Phenotypes		Number observed	Number expected		
			Ratio	Including albinos	Not including albinos
Non-albino, spotted-belly, normal-eyed	$++\ Sb$	5	3	3.375	5.625
Non-albino, spotted-belly, with ocular tumor	$+\ oc\ Sb$	3	1	1.125	1.875
Non-albino, non-spotted, normal-eyed	$+++$	5	3	3.375	5.625
Non-albino, non-spotted, with ocular tumor	$+\ oc\ +$	2	1	1.125	1.875
Total		15	8	9.000	15.000
Albino	$i\ +\ Sb$	3	8	9.000	
Do	$i\ oc\ Sb$				
Do	$i\ +\ +$				
Do	$i\ oc\ +$				
Final total		18	16	18.000	15.000

[1] $ii\ +oc\ ++ \times +i\ +oc\ Sb+$.

Hybrid B

The parents of hybrid B were spotted-dorsal platyfish-swordtail F_1 hybrids and are represented in figure 1, the third and fourth from the left on line 2. Their genetic constitutions were as follows:

$$\overset{♀♀}{+i\ +oc\ Sd+} \times \overset{♂♂}{+i\ +oc\ Sd+}$$

Sixteen of their young were reared to 12 months or over, and 2 of the 16 developed ocular tumors. In the light of the results of the previous brood, the fact that 2 of this brood had similar eye tumors suggests that genetic factors might be present for this eye abnormality. In addition to the 16 mentioned, there were 4 albino fish in the same brood, but these died within a week of birth, so that it was impossible to tell whether any of them might have developed ocular tumors. The albinos might have been in any of the 4 categories listed in table 2, but were grouped together because they appeared similar. The 4 non-albino (*1* or +) F_2 hybrid phenotypes are represented in figure 1 and their frequencies indicated on the chart. The albinos are noted on the same chart.

In table 2 from the results obtained in F_2, the value of χ^2 was 2.159, and of P 0.70; these calculations were derived by including the four inviable albinos. When the albinos were not counted, the value of χ^2 was 1.822, and of P 0.62. Each denotes significance.

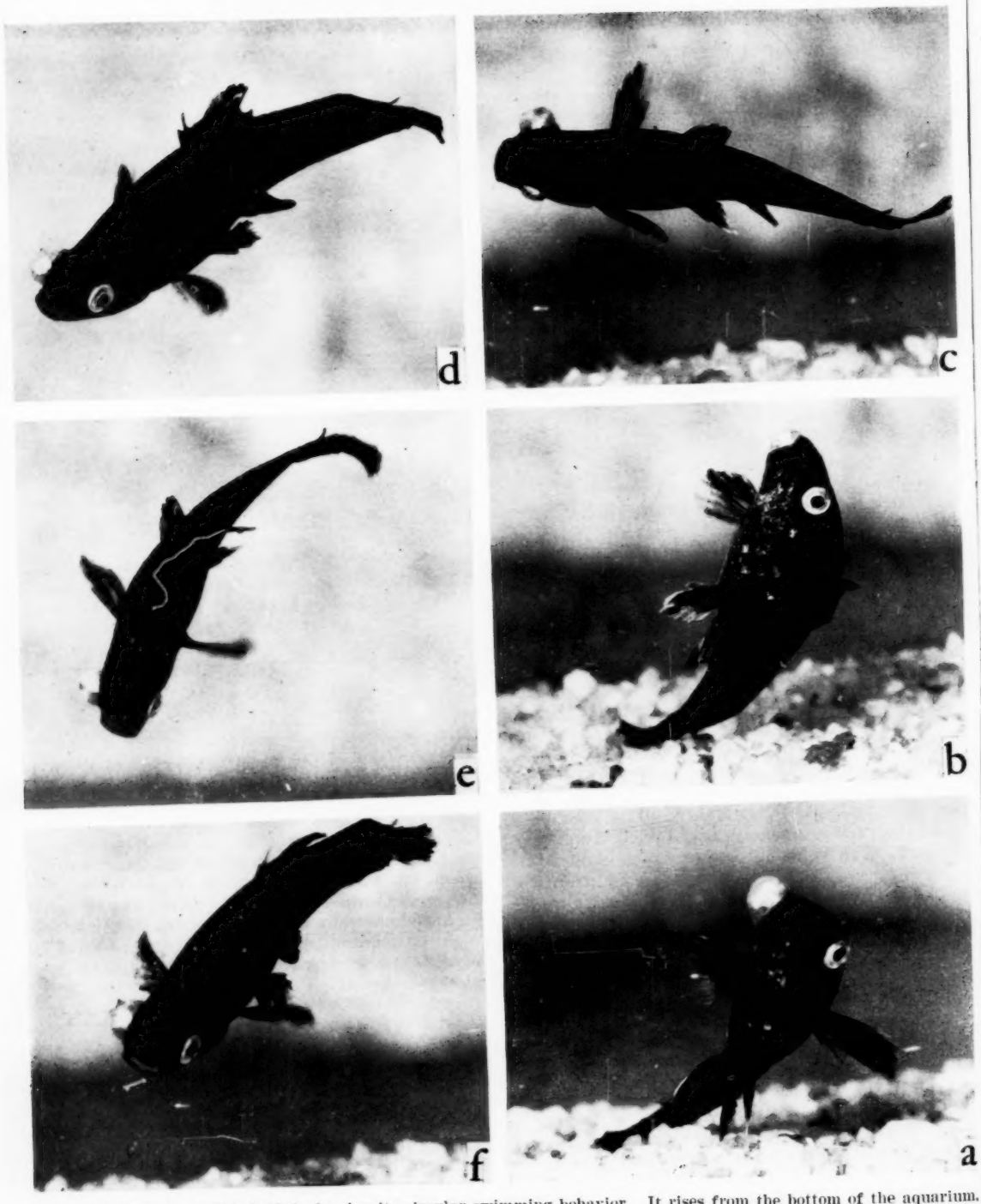

FIGURE 3.—Six views of hybrid A showing its circular swimming behavior. It rises from the bottom of the aquarium, *a*, to the position, *b*, swims forward for a short distance, *c*, and then turns downward, *d*, *e*, and approaches the sand again, *f*. Note the exophthalmic right eye, which is blind. The fish swims with its good left eye constantly toward the center. (Photographs by Sam Dunton, staff photographer of the New York Zoological Society.)

TABLE 2.—*Number and frequencies of the observed and expected phenotypes based upon the assumed constitution of the parents*[1] *of hybrid B*

Phenotypes		Number observed	Number expected		
			Ratio	Including albinos	Not including albinos
Non-albino, spotted-dorsal, normal-eyed	+ + *Sd*	11	27	8.44	9
Non-albino, spotted-dorsal, with ocular tumor	+ *oc Sd*	1	9	2.81	3
Non-albino, non-spotted, normal-eyed	+ + +	3	9	2.81	3
Non-albino, non-spotted, with ocular tumor	+ *oc* +	1	3	.94	1
Total		16	48	15.00	16
Albino	*i* + *Sd*	4	16	5.00	
Do	*i oc Sd*				
Do	*i* + +				
Do	*i oc* +				
Final total		20	64	20.00	16

[1] *+i +oc Sd+ × +i +oc Sd+.*

HYBRID C

Hybrid C, which had an ocular tumor, was described in detail previously (*1*). It was a platyfish-swordtail hybrid albino, bred in 1945 and presented to us by Fred Flathman, a private aquarist. In 1941 he had also presented us with a stock of pure albino swordtails, and all our albinos were directly traceable to these fish.

RELATION OF THE OCULAR-TUMOR FACTOR (*oc*) TO ALBINISM (*i*)

Since the paper reporting 7 genetically related fish with ocular tumors was submitted for publication, 3 additional specimens with similar eye abnormalities were found. Two of them were pure albino swordtails (182–2, and 182–11); the third, which was a golden, black-eyed, swordtail hybrid (192[2]), had an albino grandparent. Thus, with the exception of 1 pygmy swordtail and 1 wild platyfish, 10 fish with ocular tumors were traceable to albinos or offspring of albinos. Yet it was obvious that the albino gene was not responsible for ocular disturbance either in the heterozygous (*Ii*) or in the homozygous recessive (*ii*) state. However, it appeared from consideration of all the available data that the gene for albinism (*i*) was associated with the postulated gene for ocular tumors (*oc*).

If the foregoing genic association has reality, then the gene for ocular tumor produces its result as a homozygous recessive (*ococ*) but only in conjunction with the albino gene either in its heterozygous state (*Ii*) or as a homozygous recessive (*ii*). The analysis of the genetic background of these ocular tumors is difficult, owing to the prevalence of sterility in these fish, the production of small broods from a small fraction of the fertile ones, and of inviability of the albino fish in the broods produced. An additional difficulty in this study is the fact that these ocular tumors are visibly recognizable only when the fish reach a relatively old age. Most of them were 18 months old or older, yet the fish reach sexual maturity at about 8 months. Their average life span is about 2 to 2½ years.

DISCUSSION

Schlumberger and Lucké (*4*) in a monograph on tumors in cold-blooded vertebrates, which the author was kindly permitted to read recently, referred to a paper by Jahnel (*5*) on ocular tumors in swordtails. In his paper, hitherto unavailable, Jahnel described a small-cell sarcoma in each of six fish, some of which had a tumor in one eye, while others had two exophthalmic eyes. The tumors were aggressive, growing with great infiltrative powers. Most of the cells involved were small and round, but some of them were spindle-shaped. Jahnel suggested that the pathologic growths had their origin in the "Corpus chorioideale." These details were confirmed in our studies. Neither he nor we found bacteria within the ocular tumors, nor did any of us succeed in infecting normal fish by allowing them to stay for a long time within the waters of the same aquarium where tumorous fish lived.

Jahnel suggested, in view of these negative results with infectious organisms and filterable substances, that hereditary factors were probably involved. He learned from an aquarist, who bred these fish, that the swordtail parents were normal, but 9 of their 17 offspring were tumorous. He recalled the work of Häussler, Kosswig, and Breider in the experimental production of integumentary melanomas in platyfish-swordtail hybrids by genetic methods in support of the possibility that the ocular tumors might be hereditary, too. Although Jahnel's studies and our own were made independently and with apparently unrelated stocks of xiphophorin fishes, there is a remarkable parallelism in the subject matter and in the conclusions drawn. A specific genetic interpretation of the available data is now presented.

We have shown that ocular tumors may appear in hybrids carrying the dominant spotted-belly (*Sb*) gene, or the spotted-dorsal (*Sd*) gene, or the recessive, neutral-color (+) gene. Jahnel indicated that red (*R*) swordtails may also develop ocular tumors. Since other fish in the same broods had similar genes and were free of ocular tumors, none of the genes mentioned, *Sb*, *Sd*, *R*, or +, are essential for ocular-tumor growth. Jahnel referred to some of the red swordtails that he obtained as red *Xiphophorus hellerii*, but according to the recent studies of Gordon (*6*) there are no

naturally occurring red swordtails in their native habitat in Mexican waters. Many of the domesticated red platyfish-swordtail hybrids seem to have all the external attributes of pure swordtails except for their red coloring, the color gene for which was obtained by introgressive hybridization from *Platypoecilus maculatus*. Of the six tumorous fish that Jahnel studied, half were males and half females, and we are able to confirm the point that ocular tumors occur in both sexes.

In studying our genetic data, we noticed that all the fish that developed ocular tumors were either albinos or had albino parents or grandparents. From this observation, it is suggested that the albino gene may be involved in eye-tumor formation, but it is not the primary factor. It is suggested also that another autosomal gene, *oc* for ocular tumor, is the primary factor. In our stocks, the complementary action of the double recessive, *ococ*, in combination with one albino gene as $\frac{oc}{oc}\ \frac{i}{i}$ or $\frac{oc}{oc}\ \frac{i}{I}$ may account for the genetic ratios observed.

Further data may indicate that the *i* gene need not be present for the development of ocular tumors. Jahnel did not mention albinism in the parental stocks of his fish. Nor could the presence of the *i* gene be attributed to the pygmy swordtail and the common platyfish with ocular tumors described in a former paper (*1*). It may be that in some stocks, particularly in the albinos, the ocular-tumor gene *oc* may be associated with the albino gene *i* in producing the abnormality.

These studies give additional weight to a recent statement by Heston (*7*) that all tumors cannot be grouped as a single character controlled by the same gene or complex of genes and that it is necessary to consider the different types of tumors as different characters or different diseases, the inheritance of which probably involves the action of different genes. Kosswig (*8*), Gordon (*9; 10, pp. 367–375*) and Gordon and Smith (*11*) have shown that the development of melanoma of the skin in platyfish-swordtail hybrids is controlled by at least three dominant genes, one of which is sex-linked. In the present study, the development of another neoplasm, an ocular tumor, appears to be controlled by the interaction of two complementary autosomal genes both of which are recessive.

SUMMARY

Most of the instances of ocular tumors in fishes under observation of the writer were albinos or had albino parents or grandparents.

The gene for albinism *i* is not the primary factor for ocular-tumor development, but another recessive factor *oc* appears to be.

Ocular tumors were observed also in fishes in which albinism was not involved. This fact suggests that in producing the exophthalmic melanoma, the occular gene *oc* in some stocks may be associated with the *i* gene for albinism, whereas in other stocks this association may not exist.

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STUDIES ON THE INFLUENCE OF AGE ON THE LATENT-PERIOD RESPONSE OF CHICKENS TO THE AGENT OF CHICKEN TUMOR I[1]

By VERNON T. RILEY, *assistant biologist*, DOROTHY CALNAN, *junior bacteriologist*, and W. RAY BRYAN, *principal biologist*, *National Cancer Institute, National Institute of Health, United States Public Health Service*[2]

INTRODUCTION

Results of previous investigations (*1*, *2*) have shown that the latent periods of tumors induced with subcutaneous injections of the agent of chicken tumor I (*3*) may be used as criteria of biological activity for quantitative studies on this agent. Because of the relatively large numbers of test animals that are required in quantitative experiments, and because of the saving in cage space that comes with the use of young animals, it is desirable to use as young chickens as possible in routine tests or bioassays. The present studies were undertaken to determine the relative susceptibilities of chickens at the extreme limits of the age range that has been found most desirable for routine work, namely, 3 to 10 weeks at time of inoculation. In addition, chickens a few days of age have been studied to determine whether their responses differ significantly from those of the older age groups which have been more generally employed.

MATERIALS AND METHODS

Tumor Agent

Highly active suspensions of tumor agent were prepared from frozen tumor tissue ($-50°$ to $-60°$ C.) by extraction with 0.9-percent sodium chloride solution after homogenization in a Waring blender. The suspensions were partially purified by differential contrifugation (for principles, see Claude (*4*) and Beard, Bryan, and Wyckoff (*5*)) and filtration through celite (No. 503).[3] The agent was twice sedimented by high-speed centrifugation[4] and washed with phosphate buffer, pH 7.0, 0.005M. The second sedimentation pellets, obtained from a total of 10 gm. of tissue, were resuspended in 30 ml. phosphate buffer and filtered through celite columns (each 10-ml. volume was filtered through a column 8 mm. in diameter and 50 mm. in length; larger proportions of agent suspension to celite resulted in less clear filtrates). The filtrates were faintly blue in color, opalescent, and contained a higher amount of active agent per unit of nitrogen than had been obtained in this laboratory with any other method of purification. Materials obtained in this way do not represent completely pure agent since particles of at least two different population sizes and some background material are distinguishable under the electron microscope. Suspensions of agent prepared as described were employed as the starting material in the preparation of serial tenfold dilutions which were used in testing the chicken responses. Phosphate buffer, the same as already described and containing 2 percent normal rabbit serum, was used in making the serial dilutions.

Chickens

The chickens were of the New Hampshire Red breed and were hatched and raised in this laboratory. There were four different age groups, each representing the simultaneous hatch of a common lot of eggs. The ages at time of inoculation of chickens in the different age groups were 4 days, 3 weeks, 4 weeks, and 10 weeks. All chickens were taken at random without regard to sex.

Design of Experiments

Each of the experiments reported herein was for the purpose of comparing the responses of chickens of 2 different age groups to a common preparation of tumor agent. The experiments were designed so that a dose-response curve could be obtained for each age group. The positions and slopes of the respective curves were then employed to evaluate the relative susceptibilities of the chickens in the different age groups. For this purpose, each age group, which was composed of 60 chickens, was subdivided into 6 dose groups of 10 chickens each. All 10 chickens in a given dose group were injected subcutaneously with the same dilution of agent at 4 different sites, in both wings and both sides of the breast. The volume, 0.2 ml., of the inoculum was constant for all dilutions and all sites. The concentration of tumor agent is given as an equivalent in terms of the logarithm of the number of grams, per inoculum, of tumor tissue originally extracted; the concentrations are, therefore, of relative significance only and are not comparable from experiment to experiment. Examinations of chickens for tumors were carried out as previously described (*2*).

All recorded latent periods were converted to their reciprocals ($\times$ 100) for the purpose of statistical analysis (*1*, *2*). The responses at the four sites on each chicken were averaged to obtain the chicken response (*1*), and the chicken responses were analyzed by the method of analysis of vari-

[1] Received for publication October 7, 1946.
[2] The authors are indebted to Dr. C. I. Bliss, of the Connecticut Agricultural Experiment Station, for his guidance in the application of established statistical techniques to data of the type presented herein.
[3] Obtained from the Johns-Manville Co., Philadelphia.
[4] The first high-speed-sedimentation run was for 1 hour in a batch bowl of a Sharples centrifuge, type T-66-24, spinning at a rate of 48,000 r. p. m.; the second was for 1 hour in a Multispeed head of an International centrifuge, No. 2, spinning at a rate of 18,000 r. p. m.

ance (*6*, *7*) to test for differences between age groups.

RESULTS

Experiment 1

In experiment 1, the responses of 10-week-old chickens were compared with those of chickens 3 weeks of age at the time of inoculation. Although most of the experiments carried out in this laboratory have been with 3- or 4-week-old chickens, some lots have grown older before they were used, and the age has ranged as high as 8 or 10 weeks. It was desired to know whether 10-week-old chickens differ appreciably in their susceptibility to the agent of chicken tumor I from those more generally employed, i. e., 3 or 4 weeks, and whether chickens over a wide age range, e. g., 3 to 10 weeks, might be used in a single experiment where large numbers of test animals are required. (It is less desirable to use chickens more than 8 to 10 weeks of age at the time of inoculation since by the end of the 35- or 40-day observation period which follows, they are large, and an excessive amount of cage space is required to carry them through the experiment.)

The design of the experiment is given in the preceding section. The chicken responses are listed according to age and dose groups in table 1, and analyses of variance are given in table 2. In neither age group was the deviation from a linear regression significant as compared with the variation within dose groups. Linear-regression lines may therefore be employed for comparing the two sets of data. The observed dose-group means and the computed regression lines are shown in figure 1. Tests to determine whether the small differences between slopes and between positions of the respective regression lines were significant were also carried out by analysis of variance. The results are summarized in table 3. The error term for comparing the two age groups was computed from the variation of dose-group means about the calculated regression lines, since the variance for within dose variation was not homogeneous.[5] The analysis showed no significant differences between the two age groups, either in positions or in slopes of the regression lines. It is concluded, therefore, that any difference between the responses of test animals 3 weeks of age and 10 weeks of age is within the limits of error of the present experiment and is less than can be detected with groups of 60 animals. No serious error should be introduced, therefore, by differences in the ages of test animals within the limits 3 to 10 weeks.

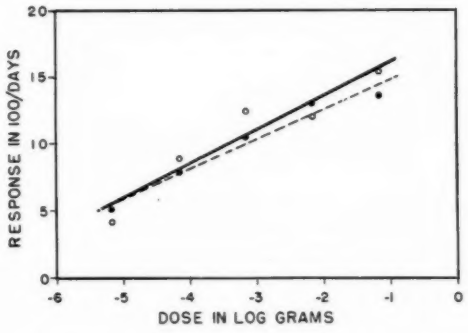

FIGURE 1.—Dose-response curves of experiment 1. Open circles and solid line, data of 3-week-old chickens; closed circles and broken line, data of 10-week-old chickens.

[5] In the instance of the present data, the conclusions regarding statistical significance are the same whether the variation of groups about regression lines or the variation of chickens within dose groups is used as the error term. Since the variance of individual chicken responses is frequently heterogeneous whereas that of group responses is usually homogeneous (*1*), there is less likelihood of encountering a degree of heterogeneity sufficient to invalidate the conclusions based upon statistical tests, if the latter variance is used for testing the significance of differences between experimental groups of chickens (e. g., different age groups, or groups kept under different experimental regimens). This amounts to the use of an average of several chicken responses instead of an individual chicken response as the unit variate and entails a loss in sensitivity of statistical tests due to a reduction in degrees of freedom. However, this loss can be compensated for, and a desired degree of accuracy can be maintained by increasing the number of chickens (and dose groups) in each experimental group. Until it is possible to reduce the heterogeneity of individual chicken responses, the authors feel that it is preferable to use the variance of dose-group responses (and fewer degrees of freedom), in comparing different experimental groups of chickens, even at the cost of a severalfold increase in the number of test animals, rather than to run the risk of interpreting differences arising from heterogeneous data as real differences.

TABLE 1.—*Individual and group responses of experiment 1*

Age groups	Dose in log grams per inoculum	[1]−6.176	−5.176	−4.176	−3.176	−2.176	−1.176
3 weeks	Responses in 100/days of individual chickens	0	4.250	7.950	12.925	13.175	14.875
		0	2.575	10.550	10.360	0	15.400
		3.825	3.050	8.150	14.350	13.550	18.175
		0	10.075	0	11.350	14.725	14.075
		4.625	5.050	10.350	12.500	[2]	13.450
		0	3.225	16.700	8.600	19.775	13.825
		1.525	2.825	12.650	11.000	13.975	16.375
		0	0	10.100	14.875	4.550	15.400
		1.725	4.550	2.175	14.725	13.775	16.150
		0	7.150	10.900	13.825	14.875	16.900
	Dose-group-mean response		4.2750	8.9525	12.4450	12.0444	15.4625
10 weeks	Responses in 100/days of individual chickens	0	5.825	7.950	8.950	14.350	15.625
		0	1.725	8.300	13.075	10.550	17.350
		1.300	4.075	10.100	10.200	11.350	12.150
		0	10.300	6.425	5.125	13.300	9.825
		0	1.050	10.625	10.975	11.750	13.825
		0	4.050	3.900	12.650	13.300	13.975
		0	8.025	2.825	19.650	13.025	14.575
		2.100	9.500	9.100	11.700	12.500	11.850
		0	0	9.550	11.900	12.225	17.650
		0	6.175	9.475	.800	17.975	9.050
	Dose-group-mean response		5.0725	7.8250	10.5025	13.0325	13.5875

[1] Omitted from statistical analyses since fewer than 50 percent of chickens developed tumors. (*See (1).*)
[2] Test chicken died an accidental death the day following inoculation.

TABLE 2.—*Analysis of variance; data of separate age groups of experiment 1*

Variation	3 weeks				10 weeks			
	Sum of squares	Degrees of freedom	Mean square	F	Sum of squares	Degrees of freedom	Mean square	F
Between dose groups	716.4534	4	179.1133	[1] 12.605	513.2954	4	128.3239	[1] 11.250
Linear regression	647.8741	1	647.8741	[1] 45.593	494.5064	1	494.5064	[1] 43.351
Deviation from linear regression	68.5793	3	22.8598	[2] 1.609	18.7890	3	6.2630	[2] .549
Within dose groups (experimental error)	625.2365	44	14.2099		513.3112	45	11.4069	
Total	1,341.6899	48			1,026.6066	49		
Correction	5,513.0625	1			5,004.0008	1		

[1] Significant at 1-percent level.
[2] Not significant.

TABLE 3.—*Analysis of variance; combined data of 3-week and 10-week age groups of experiment 1*

Variation	Sum of squares	Degrees of freedom	Mean square	F
Between positions of regression lines	10.5155	1	10.5155	[1] 0.722
Effect of combined slope	1,136.8078	1	1,136.8078	[2] 78.070
Between slopes of regression lines	5.5727	1	5.5727	[1] .383
Error (deviation from linear regression)	87.3683	6	14.5614	

[1] Not significant.
[2] Significant at 1-percent level.

EXPERIMENT 2

Experiment 2 was for the purpose of comparing the responses of 4-day-old chickens with those of a group 4 weeks old. The biological responses are listed in table 4 according to age of chickens and dose of agent. The dose-group means are plotted against logarithm of dose in figure 2. The align-

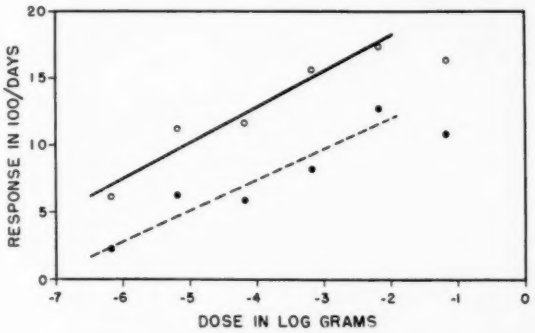

FIGURE 2.—Dose-response curves of experiment 2. Open circles and solid line, data of 4-week-old chickens; closed circles and broken line, data of 4-day-old chicks.

ment of the plotted points suggested a flattening of both dose-response curves at the highest dose level (—1.176 log grams). The suspicion that the responses might be approaching a biological limit (cf. *8*) was further supported by an analysis of variance in which the deviations from linear in the case of 3-week-old chickens approached very closely, although they did not exceed, the limits (5 percent) of chance variation. The highest dose group was therefore omitted from both series, and the data were reanalyzed. The results are shown in table 5. The best fitting straight lines to the restricted data are shown in figure 2. It is evident that the approximations to linear regressions are well within the limits of chance variation (table 5) and are sufficiently representative of the trends of the plotted points (fig. 2) to allow a comparison of the two age groups on a basis of linear dose-response curves. The comparison, by means of analysis of variance, is summarized in table 6.

TABLE 4.—*Individual and group responses of experiment 2*

Age groups	Dose in log grams per inoculum	−6.176	−5.176	−4.176	−3.176	−2.176	−1.176
4 weeks	Responses in 100/days of individual chickens	6.375	12.700	0	14.230	15.400	18.400
		4.525	10.700	13.600	15.475	17.650	18.400
		10.925	14.725	0	16.150	15.400	13.725
		0	9.425	15.400	14.875	17.650	16.150
		7.150	12.700	14.500	16.900	21.475	15.100
		12.175	10.925	13.600	14.500	17.650	16.900
		3.075	7.875	14.500	15.400	17.650	17.650
		8.850	12.550	15.400	17.650	16.150	12.925
		0	13.975	15.100	13.600	18.400	17.650
		8.300	6.275	13.600	16.900	16.375	16.900
	Dose-group-mean response	6.1375	11.2350	11.5700	15.5650	17.380	16.380
4 days	Responses in 100/days of individual chickens	2.400	2.175	10.025	13.450	13.975	7.875
		2.900	12.150	11.800	11.250	12.175	5.525
		0	9.225	0	3.975	14.875	10.100
		0	4.950	12.900	9.575	11.475	7.300
		1.325	8.075	7.550	15.675	2.950	12.700
		4.675	0	0	1.575	13.450	11.250
		3.950	11.625	0	12.500	14.500	13.600
		0	0	8.850	0	15.850	15.250
		0	7.775	7.350	2.900	16.750	11.475
		7.125	(1)	0	11.250	10.700	14.350
	Dose-group-mean response	2.2375	6.2194	5.8475	8.2150	12.6700	10.9425

[1] Died 1 day after inoculation.

TABLE 5.—*Analysis of variance; restricted data of separate age groups of experiment 2*

Variation	3 weeks					10 weeks				
	Sum of squares		Degrees of freedom	Mean square	F	Sum of squares		Degrees of freedom	Mean square	F
Between dose groups	760.8014		4	190.2004	[1] 14.109	581.6854		4	145.4214	[1] 7.117
Linear regression		719.0428	1	719.0428	[1] 53.337		524.1510	1	524.1510	[1] 25.651
Deviation from linear regression		41.7586	3	13.9195	[2] 1.032		57.5344	3	19.1781	[2] .939
Within dose groups (experimental error)	606.6528		45	13.4812		899.0767		44	20.4336	
Total	1,367.4542		49			1,480.7621		48		
Correction	7,660.1253		1			2,438.5960		1		

[1] Significant at 1-percent level.
[2] Not significant.

TABLE 6.—*Analysis of variance; combined restricted data of ½-week and ½-day age groups of experiment 2*

Variation	Sum of squares	Degrees of freedom	Mean square	F
Between positions of regression lines	714.6061	1	714.6061	[1] 43.182
Effect of combined slope	1,235.9997	1	1,235.9997	[1] 74.688
Between slopes of regression lines	7.1941	1	7.1941	[2] .435
Error (deviation from linear regression)	99.2930	6	16.5488	

[1] Significant at 1-percent level.
[2] Not significant.

Analysis of variance revealed no significant difference between the slopes of the two dose-response curves, but did indicate a highly significant difference between their relative positions (i. e., the vertical distance between the two curves is much greater than would be expected due to chance alone). The discrepancy in positions is of the magnitude that would be expected between two groups of chickens of equal susceptibility which had been inoculated with separate tumor-agent preparations differing by about a hundredfold in their relative potencies. On a basis of the quantity

of the agent that might be expected to produce a response in 4-day-old chicks equal to that in chickens 4 weeks of age, therefore, the younger animals may be said to be only about one one-hundredth as reactive, or as susceptible, as the older animals.

DISCUSSION

Although the slight differences between the dose-response curves for 3- and 10-week-old chickens observed in experiment 1 could have resulted from chance variation alone, the existence of small, but real, differences correlated with age cannot be ruled out. The present results indicate that if any dependency upon age, within the 3-to-10-week age period, is present, it is negligible as compared with experimental error due to variations among chickens of the same age. It should be possible, therefore, to pool chickens of different ages, within these limits, for use in quantitative experiments without danger of appreciably increasing the experimental error, particularly if the animals are drawn at random from the pool for assignment to the different test groups.

The results of experiment 2 show that chicks as young as 4 days at the time of inoculation differ significantly from 4-week-old chickens in their response to the agent of chicken tumor I and that they cannot therefore be pooled with the older animals in quantitative experiments. Because of their weaker response, very young chicks are less desirable for routine tests and bioassays than are chickens 3 to 10 weeks of age.

The reasons for the weaker response of 4-day-old chicks cannot be definitely stated at the present time. The most likely explanation on a basis of present knowledge is that this result represents another manifestation of the phenomenon described by Andrewes (*9*) and Duran-Reynals (*10*), namely, that neutralizing antibodies for the agent of chicken tumor I (Rous sarcoma virus) may be found in the serums of very young "normal" chicks. "Natural" antibodies for the Rous sarcoma agent have been encountered in the serums of apparently normal adult fowls 3 months of age or older, by many investigators[6] (*9–13*). Andrewes (*9*) studied the phenomenon in young chicks and found that it persisted, in detectable amounts, for only about 2 weeks after hatching. He produced evidence also which indicated that the presence of transient antibodies in young chicks was correlated with their presence in "naturally" resistant mothers, and that the antibodies were transmitted from mother to chick through the egg yolk.

If the present findings do represent the action of humoral suppressing substances, or antibodies, of the type described by Andrewes and Duran-Reynals, the possibility exists that part, if not all, of the heterogeneity found in the responses of chickens to the agent of chicken tumor I (*1*) may be owing to the action of varying amounts of such substances which persist in the bodies of test animals for varying periods.

SUMMARY

In two experiments carried out with quantitative biological techniques, studies were made of the latent-period response of chickens of different ages to the agent of chicken tumor I. No significant difference was found between the response of 3-week- and 10-week-old chickens. Four-day-old chicks, however, developed tumors at a significantly slower rate and in fewer instances (at low dose levels) than did the 4-week-old chickens with which they were compared.

A possible interpretation of the latter finding is discussed.

[6] Rous, P.: Personal communication quoted by Andrewes (*9*)

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EFFECT OF CYSTINE PER SE ON THE FORMATION OF HEPATOMAS IN RATS FOLLOWING THE INGESTION OF *p*-DIMETHYLAMINOAZOBENZENE [1]

By FLORENCE R. WHITE, *biochemist*, and JULIUS WHITE, *principal biochemist*, with the technical assistance of PHILIP W. DAVID, *National Cancer Institute, National Institute of Health, United States Public Health Service*

INTRODUCTION

We previously reported (*1*) that the restriction of cystine in rat diets containing *p*-dimethylaminoazobenzene resulted in a prolongation of the latent period and a possible but not certain reduction in the ultimate total incidence of hepatic tumors. In this experiment, the animals were fed ad libitum, and those in the cystine-restricted group grew less than did those in the cystine-supplemented group. While the effects observed might have been due to the deprivation of cystine, they could be attributed to some other factor, such as the total intake of *p*-dimethylaminoazobenzene.

The present paper describes an experiment in which the paired-feeding technique was employed to keep the food intake, and consequently the dye intake of the rats on the low-cystine and the cystine-supplemented diets equal. As far as we know, this is the first attempt made to study the effect of dietary constituents on carcinogenesis by the paired-feeding technique.

EXPERIMENTAL PROCEDURE AND RESULTS

Forty-eight pairs of litter-mate Osborne-Mendel-strain rats of the same sex, weighing 70–90 gm., were placed in individual, raised-screen platform cages. In this way the amount of food spilled could be determined, and the exact daily food intake could be calculated. One member of each pair, referred to as the low-cystine animal, ingested a diet ad libitum, composed of the following: casein, 6 percent; cornstarch, 50.94 percent; Crisco, 24 percent; dextrose, 15 percent; inorganic salts, 4 percent; and *p*-dimethylaminoazobenzene, 0.06 percent. In addition, each animal received a daily supplement of 400 mg. of dried brewers' yeast, and a weekly supplement of 0.5 ml. of cod-liver oil. The second member of each pair received a similar diet, except that 0.5 percent *l*-cystine was substituted for 0.5 percent of the starch. Rats of the latter group, referred to as the cystine-supplemented group, were not allowed to eat ad libitum, but each rat was given that quantity of food consumed the previous day by its litter mate.

Inasmuch as the rats of the cystine-supplemented group would have consumed more food had they been allowed to eat ad libitum, they were restricted in total food and hence calories. Accordingly, a third group of animals was set up. Fifteen rats, which were litter mates of 15 of the pairs employed in this experiment, were placed on the cystine-supplemented, *p*-dimethylaminoazobenzene diet; and as soon as they consumed their daily allotment of this diet, they were allowed to eat, without restriction, the same cystine-supplemented diet without *p*-dimethylaminoazobenzene. Hence all 3 groups ingested exactly the same amount of dye.

Table 1 contains a composite of the data obtained. Of the 48 pairs, 45 of the rats receiving the low-cystine diet ad libitum developed tumors earlier than did their litter mates fed the cystine-supplemented, restricted diet. Five rats of the third group developed hepatomas earlier than did their litter mates ingesting the other two diets, while 7 developed tumors later. The remarkably high incidence of hepatic tumors in all 3 groups is noteworthy. The ad libitum group (restricted in cystine) developed tumors earlier than did the other groups. Over 95 percent of the tumors arose in 200 days. The restricted group (high-cystine) developed tumors much later, about 41 percent of the tumors arising between 155 and 215 days, and about 35 percent arising between 215 and 335 days, and 23 percent between 335 and 646 days. The third group all developed hepatic tumors also; 40 percent developed tumors between 156 and 215 days, and 60 percent between 275 and 395 days. The high incidence of tumors occurring later may be attributed to the smaller proportion of dye consumed per gram of food ingested when computed in relation to body weight.

[1] Received for publication October 8, 1946.

TABLE 1.—*Number of animals with hepatic tumors*

Regimen	Effective number	Days													Mean latent period	Percentage of tumors
		96–125	126–155	156–185	186–215	216–245	246–275	276–305	306–335	336–365	366–395	396–425	426–485	486–646		
															Days	
Ad libitum [1]	48	1	16	20	9	1	1								171	100
Restricted [2]	48			8	12	7	2	2	6		2	1	2	6	248	100
Restricted plus [3]	15			4	2			2	3	2	2					100

[1] Ad libitum, allowed to consume low-cystine, *p*-dimethylaminoazobenzene diet without restriction.
[2] Restricted, allowed to consume high-cystine, *p*-dimethylaminoazobenzene diet, in amount equivalent to that eaten by its litter mate ad libitum.
[3] Restricted plus, allowed to consume same diet as the one restricted in amount and then given the basal diet without the dye ad libitum.

The amount of dye ingested, based on per unit of body weight, was calculated for each pair at the time when a tumor first appeared in any given pair. The average value for the low-cystine group was 0.025 mg. per gram of body weight; for the cystine supplemented group, 0.0144 mg. per gram of body weight; and for the group on the cystine-supplemented diet plus the basal diet, 0.0076 mg. per gram of body weight. Thus, the low-cystine group consumed approximately 40 percent more dye than did the high-cystine group based on per unit of body weight.

In our previous experiment (*1*), in which both groups ate ad libitum, the high-cystine group developed tumors more rapidly. The high-cystine group consumed more food and hence more of the carcinogen. In fact, the animals ingesting the high-cystine diet consumed approximately 35 percent more on the basis of unit of body weight than did the animals ingesting the low-cystine diet.

Table 2 is a composite of the average body weights of all groups of animals at the time that a tumor first appeared in any given pair. Animals on the restricted diet, and on the restricted plus (cystine-supplemented) gained more weight since the diet was better suited for growth.

TABLE 2.—*Mean body weights and range of body weights when tumors first appeared*

Regimen	Number of animals	Mean body weight [1]	Range of body weight [2]
		Grams	*Grams*
Ad libitum [1]	48	F 126	F 108–163
		M 136	M 105–162
Restricted	48	F 172	F 126–239
		M 181	M 131–234
Restricted plus	15	F 229	F 227–319
		M 284	M 214–296

F, female; M, male.
[1] See table 1 for explanation.
[2] At time tumor first appeared in any given pair.

DISCUSSION

In some earlier reports (*2, 3*), evidence was presented indicating that the oral administration of certain polycyclic hydrocarbons known to produce tumors in some animals could retard the growth of young rats ingesting a diet relatively low in cystine. The addition of 0.5 percent cystine (or methionine) to the diet still containing the hydrocarbon would result in a prompt stimulation in growth (increase in weight). Other substances, such as glycine or sodium sulfate, had no such stimulating effect on growth when added to the diet of rats ingesting the hydrocarbons. Yet in spite of the ability of cystine to overcome the growth-retarding effect of these hydrocarbons as well as *p*-dimethylaminoazobenzene (*3*), animals ingesting a diet containing adequate cystine to react stoichiometrically with the dye were still able to develop hepatic tumors with no apparent difficulty.

It is conceivable that the concentration employed in the previous experiment, as well as in this one, far exceeded that needed to produce hepatic tumors and that the addition of cystine in the amount used could not effect the formation of these neoplasms.

To establish the actual effect of cystine on hepatic-tumor formation by the administration of *p*-dimethylaminoazobenzene, it would be necessary in the presence of adequate cystine, to administer various amounts of the dye for given periods.

The ability of the group fed the restricted diet (containing added cystine) to gain more weight than the group fed ad libitum (low-cystine) can probably be explained in part by referring to the work of Tepperman, Brobeck, and Long (*4*). They showed that animals trained to ingest all their food in a relatively short time will gain more rapidly and to a higher degree than will animals consuming the same amount of food over a longer period. Our ad-libitum animals had access to food at all times. However, the rats of the restricted group (cystine-supplemented) which were given an amount of food equivalent to that eaten by their litter mates the previous day, devoured their food in 5 minutes. Furthermore, the diet containing added cystine was more adaptable for growth.

The high incidence of hepatic tumors in all three groups indicates that cystine deprivation in itself probably exerts no effect on the total incidence of hepatomas caused by the ingestion of *p*-dimethylaminoazobenzene at the levels fed.

To determine the effects of various ingredients of the diet on hepatic-tumor formation, comparative studies should be made with as many variable factors as possible eliminated. It would be desirable to use litter-mate controls that are of the same sex. Food-intake data and the exact amount of carcinogen ingested by each animal should be available. The method of determining the presence of tumors should be standardized. Rusch and his groups employ laparotomy at 4 months, and the animals are killed at 6 months and the final tumor counts made. Except for the total time of analysis, which we believe to be short, this procedure affords an excellently standardized method of examination. However, it would be desirable that laparotomies be performed again at 6 months and the animals killed at 12 to 18 months. This change would give a more accurate picture and would increase the available data on latent-period effects.

Our method of tumor determination was palpation through the abdominal wall, and it may be

subject to the criticism that it does not represent the true time of onset of hepatoma.[2] But since a comparison is made on at least two groups, this method is satisfactory. If no tumor is ever palpable, the animal is allowed to live its natural life, and final examination is made at necropsy. All gross observations should be verified by histologic examination of prepared sections of the tumors.

[2] Verified by microscopic examination of stained section.

SUMMARY AND CONCLUSIONS

The effect of cystine on the production of hepatic tumors in rats of the Osborne-Mendel strain by the oral administration of *p*-dimethylaminoazobenzene was determined by the paired-feeding technique.

Cystine deprivation under these conditions probably exerts no effect on the total production of hepatomas resulting from the ingestion of the dye.

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